

## CERTIFICATION UNDER 37 CFR 1.10

09/701500

EI685061565US

Express Mail Mailing Number

29 NOV 2000

Date of Deposit

I hereby certify that this paper or fee, and any documents referred to as enclosed herein, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

Cederic Rodgers

Name of Person Mailing Application

Signature of Person Mailing Application

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TSRI 651.1

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)  
Not known yet

INTERNATIONAL APPLICATION NO.  
PCT/US99/11780

INTERNATIONAL FILING DATE  
28 MAY 1999

PRIORITY DATE CLAIMED  
29 MAY 1998

TITLE OF INVENTION

METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF ANGIOGENESIS USING TYROSINE KINASE SRC

APPLICANT(S) FOR DO/EO/US

David A. Cheresch, Brian Eliceiri and Pamela L. Schwartzberg

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ An English language translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) 35 U.S.C. 371(c)(4)) and **Power of Attorney** (Executed).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
- ☐ A SECOND or SUBSEQUENT preliminary amendment.

563 Rec'd PCT/PTO 29 NOV 2009

|  |   |                                       |
|--|---|---------------------------------------|
| US APPLICATION NO. (if known, see 37 CFR 1.5)<br><div style="font-size: 1.5em; font-weight: bold; margin-top: 10px;">09/701500</div> | INTERNATIONAL APPLICATION NO.<br>PCT/US99/11780 | ATTORNEYS DOCKET NUMBER<br>TSRI 651.1 |
|--|---|---------------------------------------|

|   |  |
|---|--|
| 14. <input type="checkbox"/> A substitute specification and claims amended to conform the translated application to US practice<br>15. <input type="checkbox"/> A change of power of attorney and/or address letter.<br>16. <input checked="" type="checkbox"/> Other items or information:<br>(a) copy of form PCT/IB/308 (Notification of Communication of International Application to the Designated Offices);<br>(b) copy of the First Page of International Publication;<br>(c) copy of International Search Report;<br>(d) copies of three (3) forms PCT/IB/306 (Notification of the Recording of a Change);<br>(e) copies of Written Opinion and International Preliminary Examination Report |  |
|---|--|

| 17. <input checked="" type="checkbox"/> The following fees are submitted:<br><br><b>BASIC NATIONAL FEE (37 CFR 1.492(A)(1)-(5)):</b><br><br>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,000.00<br><br>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00<br><br>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00<br><br>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00<br><br>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00<br><br><div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div> | <b>CALCULATIONS</b> <span style="float: right; font-size: 0.8em;">PTO USE ONLY</span>  |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
|--|--|--------------|--------------|------|--------------|----|----|---------|--------------------|---|---|---------|---|--|--|----------|---|
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).  | <div style="border: 1px solid black; height: 150px; margin-bottom: 10px;"></div> <div style="display: flex; justify-content: space-between;"> <span><b>\$690.00</b></span> <span></span> </div> <div style="display: flex; justify-content: space-between;"> <span><b>\$0.00</b></span> <span></span> </div> |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 20%;">Claims</th> <th style="width: 20%;">Number Filed</th> <th style="width: 20%;">Number Extra</th> <th style="width: 20%;">Rate</th> </tr> </thead> <tbody> <tr> <td>Total Claims</td> <td style="text-align: center;">38</td> <td style="text-align: center;">18</td> <td style="text-align: center;">\$18.00</td> </tr> <tr> <td>Independent Claims</td> <td style="text-align: center;">8</td> <td style="text-align: center;">5</td> <td style="text-align: center;">\$80.00</td> </tr> <tr> <td colspan="3">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td style="text-align: center;">\$270.00</td> </tr> </tbody> </table>   | Claims   | Number Filed | Number Extra | Rate | Total Claims | 38 | 18 | \$18.00 | Independent Claims | 8 | 5 | \$80.00 | MULTIPLE DEPENDENT CLAIM(S) (if applicable) |  |  | \$270.00 | <div style="display: flex; justify-content: space-between;"> <span><b>\$324.00</b></span> <span></span> </div> <div style="display: flex; justify-content: space-between;"> <span><b>\$400.00</b></span> <span></span> </div> |
| Claims   | Number Filed   | Number Extra | Rate         |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| Total Claims   | 38   | 18           | \$18.00      |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| Independent Claims   | 8  | 5            | \$80.00      |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable)  |  |              | \$270.00     |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| <b>TOTAL OF ABOVE CALCULATIONS =</b>   | <div style="display: flex; justify-content: space-between;"> <span><b>\$1,414.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.   | <div style="display: flex; justify-content: space-between;"> <span><b>\$0.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| <b>SUBTOTAL =</b>  | <div style="display: flex; justify-content: space-between;"> <span><b>\$1,414.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).   | <div style="display: flex; justify-content: space-between;"> <span><b>\$0.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| <b>TOTAL NATIONAL FEE =</b>  | <div style="display: flex; justify-content: space-between;"> <span><b>\$1,414.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) \$40 per property   | <div style="display: flex; justify-content: space-between;"> <span><b>\$0.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
| <b>TOTAL FEES ENCLOSED =</b>   | <div style="display: flex; justify-content: space-between;"> <span><b>\$1,414.00</b></span> <span></span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
|  | <div style="display: flex; justify-content: space-between;"> <span>Amount to be refunded</span> <span>\$</span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |
|  | <div style="display: flex; justify-content: space-between;"> <span>charged</span> <span>\$</span> </div>   |              |              |      |              |    |    |         |                    |   |   |         |   |  |  |          |   |

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525 Rec'd PCT/PTO 29 NOV 2000

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|---|---|---------------------------------------|
| US APPLICATION NO. (if known, see 37 CFR 1.101)<br><b>09/701500</b> | INTERNATIONAL APPLICATION NO.<br>PCT/US99/11780 | ATTORNEYS DOCKET NUMBER<br>TSRI 651.1 |
|---|---|---------------------------------------|

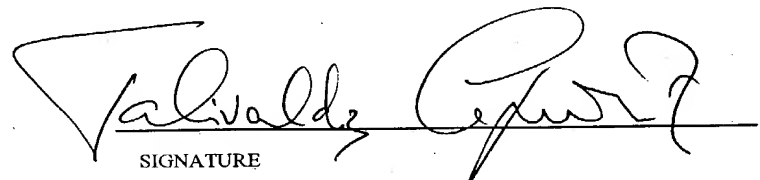
- a. ☒ A check in the amount of \$1,414.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional filing fees which may be required, or credit any overpayment to Deposit Account No. 15-0508. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Talivaldis Cepuritis

NAME

20,818

REGISTRATION NO.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Applicant(s): David A. Cheresh et al. )  
Serial No.: 09/701,500 )  
Filed: November 29, 2000 )  
For: METHODS AND COMPOSITIONS )  
USEFUL FOR MODULATION OF )  
ANGIOGENESIS USING )  
TYROSINE KINZE SRC )  
Atty. Docket No.: TSRI 651.1

COMMUNICATION

Box PCT  
Commissioner for Patents  
Washington, D.C. 20231

Sir:

Pursuant to the phone request of Mr. John Anderson of the U.S. Patent Office on November 14, 2002, enclosed is a duplicate diskette of the "Sequence Listing" to replace the diskette misplaced.

This submission does not constitute new matter and is supported in the application as filed.

Respectfully submitted,

By Deborah L. Boklund-Moran  
Deborah L. Boklund-Moran  
Foreign Patent Paralegal for  
Talivaldis Cepuritis (Reg. No. 20,818)

OLSON & HIERL, LTD.  
20 North Wacker Drive  
36th Floor  
Chicago, Illinois 60606  
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Serial No. 09/701,500 - - - - - 2

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Cedric Rodgers

(Typed or printed name of person mailing paper or fee)

*Cedric Rodgers*

(Signature of person mailing paper or fee)

JC17 Rec'd PCT/PTO 24 MAY 2001

09/701500

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Applicant(s): David A. Cheresh et al. )

Serial No.: 09/701,500 )

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For: METHODS AND COMPOSITIONS  
USEFUL FOR MODULATION OF  
ANGIOGENESIS USING  
TYROSINE KINZE SRC )

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COMMUNICATION

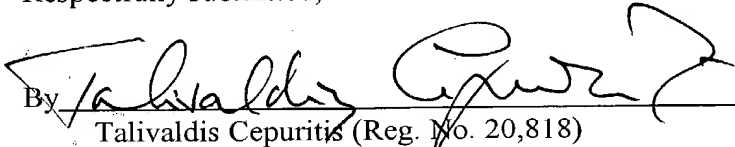
Box PCT  
Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C. 371 In the United States Designated/Elected Office (DO/EO/US) dated April 30, 2001, enclosed is a substitute paper copy and computer-readable form of the "Sequence Listing." A copy of the Patent Office Notification is returned herewith.

This submission does not constitute new matter and is supported in the application as filed. To the best of my information and belief, the sequence listing information recorded in computer-readable form is identical to the paper copy of the sequence listing.

Respectfully submitted,

By   
Talivaldis Cepuritis (Reg. No. 20,818)

OLSON & HIERL, LTD.  
20 North Wacker Drive  
36th Floor  
Chicago, Illinois 60606  
(312) 580-1180

Serial No. 09/701,500 - - - - - 2

**CERTIFICATION UNDER 37 CFR 1.10**

"Express Mail" mailing label number: EL 841695176 US

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Cedric Rodgers

(Typed or printed name of person mailing paper or fee)

Cedric Rodgers

(Signature of person mailing paper or fee)

METHODS AND COMPOSITIONS USEFUL FOR MODULATION  
OF ANGIOGENESIS USING TYROSINE KINASE SRC

Reference to Related Application

5        This application claims the benefit of U.S. Provisional Application Serial  
No. 60/087,220 filed May 29, 1998, which is incorporated by reference, as are all  
references cited herein.

Technical Field

10        The present invention relates generally to the field of medicine, and relates  
specifically to methods and compositions for modulating angiogenesis of tissues  
using the protein tyrosine kinase Src, variants of Src, and nucleic acids encoding  
them.

Background

15        Angiogenesis is a process of tissue vascularization that involves the growth of  
new developing blood vessels into a tissue, and is also referred to as neo-  
vascularization. The process is mediated by the infiltration of endothelial cells and  
smooth muscle cells. The process is believed to proceed in any one of three ways:  
the vessels can sprout from pre-existing vessels, de-novo development of vessels  
can arise from precursor cells (vasculogenesis), or existing small vessels can  
20        enlarge in diameter. Blood et al., Bioch. Biophys. Acta, 1032:89-118 (1990).

Angiogenesis is an important process in neonatal growth, but is also important  
in wound healing and in the pathogenesis of a large variety of clinical diseases  
including tissue inflammation, arthritis, tumor growth, diabetic retinopathy,  
macular degeneration by neovascularization of the retina and like conditions.  
25        These clinical manifestations associated with angiogenesis are referred to as  
angiogenic diseases. Folkman et al., Science, 235:442-447 (1987). Angiogenesis  
is generally absent in adult or mature tissues, although it does occur in wound  
healing and in the corpus luteum growth cycle. See, for example, Moses et al.,  
Science, 248:1408-1410 (1990).

30        It has been proposed that inhibition of angiogenesis would be a useful therapy  
for restricting tumor growth. Inhibition of angiogenesis has been proposed by (1)  
inhibition of release of "angiogenic molecules" such as bFGF (basic fibroblast  
growth factor), (2) neutralization of angiogenic molecules, such as by use of anti-

$\beta$ bFGF antibodies, (3) use of inhibitors of vitronectin receptor  $\alpha_v\beta_3$ , and (4) inhibition of endothelial cell response to angiogenic stimuli. This latter strategy has received attention, and Folkman et al., Cancer Biology, 3:89-96 (1992), have described several endothelial cell response inhibitors, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D<sub>3</sub> analogs, alpha-interferon, and the like that might be used to inhibit angiogenesis. For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-118 (1990), Moses et al., Science, 248:1408-1410 (1990), Ingber et al., Lab. Invest., 59:44-51 (1988), and United States Patent Nos. 5,092,885, 5,112,946, 5,192,744, 5,202,352, 5,753,230 and 5,766,591. None of the inhibitors of angiogenesis described in the foregoing references involve the Src proteins.

For angiogenesis to occur, endothelial cells must first degrade and cross the blood vessel basement membrane in a similar manner used by tumor cells during invasion and metastasis formation.

It has been previously reported that angiogenesis depends on the interaction between vascular integrins and extracellular matrix proteins. Brooks et al., Science, 264:569-571 (1994). Furthermore, it was reported that programmed cell death (apoptosis) of angiogenic vascular cells is initiated by the interaction, which would be inhibited by certain antagonists of the vascular integrin  $\alpha_v\beta_3$ . Brooks et al., Cell, 79:1157-1164 (1994). More recently, it has been reported that the binding of matrix metalloproteinase-2 (MMP-2) to vitronectin receptor ( $\alpha_v\beta_3$ ) can be inhibited using  $\alpha_v\beta_3$  antagonists, and thereby inhibit the enzymatic function of the proteinase. Brooks et al., Cell, 85:683-693 (1996).

#### Summary of the Invention

The present invention is directed to modulation of angiogenesis in tissues by tyrosine kinase Src, also referred to generically herein as Src.

Compositions and methods for modulating angiogenesis in a tissue associated with a disease condition are contemplated. A composition comprising an angiogenesis-modulating amount of a Src protein is administered to tissue to be treated for a disease condition that responds to modulation of angiogenesis. The composition providing the Src protein can contain purified protein, biologically

active protein fragments, recombinantly produced Src protein or protein fragments or fusion proteins thereof, or gene/nucleic acid expression vectors for expressing a Src protein.

Where the Src protein is inactivated or inhibited, the modulation is an inhibition of angiogenesis. Where the Src protein is active or activated, the modulation is a potentiation of angiogenesis.

The tissue to be treated can be any tissue in which modulation of angiogenesis is desirable. For angiogenesis inhibition, it is useful to treat diseased tissue where deleterious neovascularization is occurring. Exemplary tissues include inflamed tissue, solid tumors, metastases, tissues undergoing restenosis, and the like tissues.

For potentiation, it is useful to treat patients with ischemic limbs in which there is poor circulation in the limbs from diabetic or other conditions. Patients with chronic wounds that do not heal and therefore could benefit from the increase in vascular cell proliferation and neovascularization can be treated as well.

Particularly preferred is the use of Src protein containing a modified amino acid sequence as described herein. Several particularly useful modified Src proteins and the expression thereof are described herein.

The present invention also encompasses a pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a viral or non-viral gene transfer vector containing a nucleic acid and a pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein, said src protein having any amino acid residue at codon 527 except tyrosine, serine or threonine.

Also envisioned is a pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a viral or non-viral gene transfer vector containing a nucleic acid and a pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein having no kinase activity.

#### Brief Description of the Drawings

In the drawings forming a portion of this disclosure:

FIG. 1 is a cDNA sequence of chicken c-Src which is the complete coding sequence with the introns deleted as first described by Takeya et al., *Cell*, 32:881-890 (1983). The sequence is accessible through GenBank Accession Number

J00844. The sequence contains 1759 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 112 and 1713.

FIG. 2 is the encoded amino acid residue sequence of chicken c-Src of the coding sequence shown in FIG. 1.

5        FIG. 3 is a cDNA sequence of human c-Src which as first described by Braeuninger et al., Proc. Natl. Acad. Sci., USA, 88:10411-10415 (1991). The sequence is accessible through GenBank Accession Number X59932 X71157. The sequence contains 2187 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 134 and 1486.

10        FIG. 4 is the encoded amino acid residue sequence of human c-Src of the coding sequence shown in FIG. 3.

FIG. 5 illustrates the activation of endogenous Src by bFGF or VEGF as described in Example 4. The top portion of the figure indicates the results of an in vitro kinase assay with the fold activation of endogenous c-Src by either bFGF and VEGF. The bottom portion of the figure is the kinase assay blot probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

15        FIG. 6 illustrates the effect of retrovirus-mediated gene expression of c-Src A on angiogenesis in the chick chorioallantoic membrane (CAM) as described in Example 4. Nine-day-old chick CAMs were exposed to RCAS-Src A (active mutated c-Src) or control RCAS-GFP (Green Fluorescent Protein; a fluorescent indicator protein) retroviruses or buffer for 72 h. The level of angiogenesis was quantified as shown in FIG. 6A with representative photomicrographs (4x) in FIG. 6B corresponding to each treatment taken with a stereomicroscope.

20        FIG. 7 illustrates the retroviral expression of c-Src A in activating vascular MAP kinase phosphorylation. FIG. 7A shows tissue extracts of 10 day-old chick CAMs that had been exposed to VEGF or PMA for 30 minutes or infected with c-Src A retrovirus for 48 hours. NT stands for no treatment. Src was immunoprecipitated from equivalent amounts of total protein extract and subjected to an in vitro immune complex kinase assay using a FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose. Aliquots of the above whole tissue lysates were also measured for endogenous ERK phosphorylation by immunoblotting with an anti-phospho-ERK antibody. FIG. 7B shows 10 day old CAMs that were infected with either mock RCAS or RCAS containing SRC A.

After two days, CAMs were dissected, cryopreserved in OCT and sectioned at 4  $\mu$ m. Sections were immunostained with an anti-phosphorylated ERK antibody (New England Biolabs), washed and detected with a goat anti-rabbit FITC-conjugated secondary antibody. Florescent images were captured on a cooled-  
5 CCD camera (Princeton Inst.)

FIG. 8 illustrates the selective requirement for Src activity during VEGF, but not bFGF-induced angiogenesis. Nine day old chick CAMs were exposed to RCAS-Src 251 or control RCAS-GFP retroviruses or buffer for 20 hours and then incubated for an additional 72 hours in the presence or absence of bFGF or  
10 VEGF. The level of angiogenesis was quantified FIG. 8A as described above, and representative photomicrographs (6x) were taken with a stereomicroscope as shown in FIG. 8B. FIG. 8C shows a blot probed with an anti-Src antibody to confirm the expression of Src 251 in transfected cells as compared to mock treatments.

FIG. 9 illustrates the results of retroviral delivery of RCAS-Src 251 to human tumors. FIG. 9A is a micrograph that shows human medulloblastoma tumor fragment infected with RCAS-GFP (RCAS-Green Fluorescent Protein) expressing GFP exclusively in the tumor blood vessels (arrowhead) as detected by optical sectioning with a Bio Rad laser confocal scanning microscope (bar=500  $\mu$ m).  
20 FIG. 9B depicts data from tumors treated with topical application of retrovirus, which were allowed to grow for 3 or 6 days after which they were resected and wet weights determined. Data are expressed as the mean change in tumor weight (from the 50 mg tumor starting weight) +/- SEM of 2 replicates. FIG. 9C depicts in representative micrographs, medulloblastoma tumors surgically removed from  
25 the embryos (bar=350  $\mu$ m). The lower panels are high magnification views of each tumor showing the vasculature of each tumor in detail (bar=350  $\mu$ m). The arrowhead indicates blood vessel disruption in RCAS-Src251-treated tumors.

FIG. 10 is a diagram illustrating a restriction map of the RCASBP (RCAS) vector construct.

#### 30 Detailed Description of the Invention

##### A. Definitions

Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues



described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide in keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR §1.822(b)(2)).

It should be noted that all amino acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues.

Polypeptide: refers to a linear array of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues.

Peptide: as used herein refers to a linear array of no more than about 50 amino acid residues connected one to the other as in a polypeptide.

Cyclic peptide: refers to a compound having a ring structure that includes several amide bonds as in a typical peptide. The cyclic peptide can be a "head to tail" homodetic cyclic peptide, or it can contain a heterodetic ring structure in which the ring is closed by disulfide bridges, lactam bridges, thioesters, thioamides, guanidino, and the like linkages.

Protein: refers to a linear array of more than 50 amino acid residues connected one to the other as in a polypeptide.

Fusion protein: refers to a polypeptide containing at least two different polypeptide domains operatively linked by a typical peptide bond ("fused"), where the two domains correspond to peptides not found fused in nature.

Synthetic peptide: refers to a chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

#### B. General Considerations

The present invention relates generally to the discovery that angiogenesis is mediated by the tyrosine kinase Src protein, and that angiogenesis can be

modulated by providing either active or inactive Src proteins for potentiating or inhibiting angiogenesis, respectively.

5 This discovery is important because of the role that angiogenesis, the formation of new blood vessels, plays in a variety of disease processes. Where tissues associated with a disease condition require angiogenesis for tissue growth, it is desirable to inhibit angiogenesis and thereby inhibit the diseased tissue growth. Where injured tissue requires angiogenesis for tissue growth and healing, it is desirable to potentiate or promote angiogenesis and thereby promote tissue healing and growth.

10 Where the growth of new blood vessels is the cause of, or contributes to, the pathology associated with a diseased tissue, inhibition of angiogenesis reduces the deleterious effects of the disease. By inhibiting angiogenesis, one can intervene in the disease, ameliorate the symptoms, and in some cases cure the disease.

15 Examples of tissue associated with disease and neovascularization that will benefit from inhibitory modulation of angiogenesis include rheumatoid arthritis, diabetic retinopathy, inflammatory diseases, restenosis, and the like. Where the growth of new blood vessels is required to support growth of a deleterious tissue, inhibition of angiogenesis will reduce the blood supply to the tissue and thereby contribute to reduction in tissue mass based on blood supply requirements.

20 Examples include growth of tumors where neovascularization is a continual requirement in order that the tumor grow beyond a few millimeters in thickness, and for the establishment of solid tumor metastases.

25 Where the growth of new blood vessels contributes to healing of tissue, potentiation of angiogenesis assists in healing. Examples include treatment of patients with ischemic limbs in which there is poor circulation in the limbs from diabetes or other conditions. Also contemplated for treatment are patients with chronic wounds that do not heal and therefore could benefit from the increase in vascular cell proliferation and neovascularization.

30 The methods of the present invention are effective in part because the therapy is highly selective for angiogenesis and not other biological processes.

As described earlier, angiogenesis includes a variety of processes involving neovascularization of a tissue including "sprouting", vasculogenesis, or vessel enlargement, all of which angiogenesis processes are effected by Src protein.

With the exception of traumatic wound healing, corpus luteum formation and embryogenesis, it is believed that the majority of angiogenesis processes are associated with disease processes. Accordingly, the present therapeutic methods are selective for the disease and do not have deleterious side effects.

5 C. Src Proteins

A tyrosine kinase Src protein for use in the present invention can vary depending upon the intended use. The terms "Src protein" or "Src" are used to refer to the various forms of tyrosine kinase Src proteins described herein, either in active or inactive forms.

10 An "active Src protein" refers to any of a variety of forms of src protein which potentiate angiogenesis. Assays to measure potentiation of angiogenesis are described herein, and are not to be construed as limiting. A protein is considered active if the level of angiogenesis is at least 10% greater, preferably 25% greater, and more preferably 50% greater than a control level where no src is added to the  
15 assay system. The preferred assay for measuring potentiation is the CAM assay using RCAS viral vector as described in the Examples in which the angiogenic index is calculated by counting branch points. A preferred active Src protein exhibits tyrosine kinase activity as well. Exemplary active Src proteins are described in the Examples, and include Src-A.

20 An "inactive Src protein" refers to any of a variety of forms of Src protein which inhibit angiogenesis. Assays to measure inhibition of angiogenesis are described herein, and are not to be construed as limiting. A protein is considered inactive if the level of angiogenesis is at least 10% lower, preferably 25% lower, and more preferably 50% lower than a control level where no exogenous Src is  
25 added to the assay system. The preferred assay for measuring inhibition is the CAM assay using RCAS viral vector as described in the Examples in which the angiogenic index is calculated by counting branch points. A preferred inactive Src protein exhibits reduced tyrosine kinase activity as well. Exemplary inactive Src proteins are described in the Examples, and include Src-251.

30 A Src protein useful in the present invention can be produced in any of a variety of methods including isolation from natural sources including tissue, production by recombinant DNA expression and purification, and the like. Src

protein can also be provided "in situ" by introduction of a gene therapy system to the tissue of interest which then expresses the protein in the tissue.

A gene encoding a Src protein can be prepared by a variety of methods known in the art, and the invention is not to be construed as limiting in this regard. For example, the natural history of Src is well known to include a variety of homologs from mammalian, avian, viral and the like species, and the gene can readily be cloned using cDNA cloning methods from any tissue expressing the protein. A preferred Src for use in the invention is a cellular protein, such as the mammalian or avian homologs designated c-Src. Particularly preferred is human c-Src.

10 D. Recombinant DNA Molecules and Expression Systems for Expression of a Src Protein

The invention describes several nucleotide sequences of particular use in the present invention. These sequences include sequences which encode a Src protein useful in the invention, and various DNA segments, recombinant DNA (rDNA) molecules and vectors constructed for expression of Src protein.

DNA molecules (segments) of this invention therefore can comprise sequences which encode whole structural genes, fragments of structural genes, and transcription units as described further herein.

A preferred DNA segment is a nucleotide sequence which encodes a Src protein as defined herein, or biologically active fragment thereof,

The amino acid residue sequence and nucleotide sequence of a preferred c-Src is described in the Examples.

A preferred DNA segment codes for an amino acid residue sequence substantially the same as, and preferably consisting essentially of, an amino acid residue sequence or portions thereof corresponding to a Src protein described herein. Representative and preferred DNA segments are further described in the Examples.

The amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the structural gene that codes for the protein. Thus, a structural gene or DNA segment can be defined in terms of the amino acid residue sequence, i.e., protein or polypeptide, for which it codes.

An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylations do not affect the coding relationship in any way.

A nucleic acid is any polynucleotide or nucleic acid fragment, whether it be a polyribonucleotide or polydeoxyribonucleotide, i.e., RNA or DNA, or analogs thereof. In preferred embodiments, a nucleic acid molecule is in the form of a segment of duplex DNA, i.e., a DNA segment, although for certain molecular biological methodologies, single-stranded DNA or RNA is preferred.

DNA segments are produced by a number of means including chemical synthesis methods and recombinant approaches, preferably by cloning or by polymerase chain reaction (PCR). DNA segments that encode portions of a Src protein can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al, *J. Am. Chem. Soc.*, 103:3185-3191, 1981, or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define the DNA segment, followed by hybridization and ligation of oligonucleotides to build the complete segment. Alternative methods include isolation of a preferred DNA segment by PCR with a pair of oligonucleotide primers used on a cDNA library believed to contain members which encode a Src protein.

Of course, through chemical synthesis, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. This method is well known, and can be readily applied to the production of the various different "modified" Src proteins described herein.

Furthermore, DNA segments consisting essentially of structural genes encoding a Src protein can be subsequently modified, as by site-directed or random mutagenesis, to introduce any desired substitutions.

### 1. Cloning a Src Gene

A Src gene can be cloned from a suitable source of genomic DNA or messenger RNA (mRNA) by a variety of biochemical methods. Cloning of these genes can be conducted according to the general methods described in the

5 Examples and as known in the art.

Sources of nucleic acids for cloning a Src gene suitable for use in the methods of this invention can include genomic DNA or messenger RNA (mRNA) in the form of a cDNA library, from a tissue believed to express these proteins. A preferred tissue is human lung tissue, although any other suitable tissue may be

10 used.

A preferred cloning method involves the preparation of a cDNA library using standard methods, and isolating the Src-encoding nucleotide sequence by PCR amplification using paired oligonucleotide primers based on the nucleotide sequences described herein. Alternatively, the desired cDNA clones can be

15 identified and isolated from a cDNA or genomic library by conventional nucleic acid hybridization methods using a hybridization probe based on the nucleic acid sequences described herein. Other methods of isolating and cloning suitable src encoding nucleic acids are readily apparent to one skilled in the art.

### 2. Expression Vectors

20 A recombinant DNA molecule (rDNA) containing a DNA segment encoding a Src protein can be produced as described herein. In particular, an expressible rDNA can be produced by operatively (in frame, expressibly) linking a vector to a src encoding DNA segment. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleic acids of a nucleotide sequences not

25 normally found together in nature.

The choice of vector to which the DNA segment is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector suitable for use in practicing the present invention is at least capable of directing the replication,

30 and preferably also expression, of a structural gene included in the vector DNA segments to which it is operatively linked.

Both prokaryotic and eukaryotic expression vectors are familiar to one of ordinary skill in the art of vector construction, and are described by Ausubel, et

al., in Current Protocols in Molecular Biology, Wiley and Sons, New York (1993) and by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, (1989). These references also describe many of the general recombinant DNA methods referred to herein.

5 In one embodiment, a suitable vector includes a procaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, those embodiments that include a procaryotic  
10 replicon also include a gene whose expression confers drug resistance to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Those vectors that include a procaryotic replicon can also include a procaryotic promoter capable of directing the expression (transcription and  
15 translation) of a structural gene in a bacterial host cell, such as E. coli, transformed therewith. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA  
20 segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pRSET available from Invitrogen (San Diego, CA) and pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those  
25 compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10  
30 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), pRc/CMV (Invitrogen, Inc.), the preferred vector described in the Examples, and the like eukaryotic expression vectors.

A particularly preferred system for gene expression in the context of this invention includes a gene delivery component, that is, the ability to deliver the gene to the tissue of interest. Suitable vectors are "infectious" vectors such as recombinant DNA viruses, adenovirus or retrovirus vectors which are engineered  
5 to express the desired protein and have features which allow infection of preselected target tissues. Particularly preferred is the replication competent avian sarcoma virus (RCAS) described herein.

Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus  
10 expression vectors, the coding sequence of a polypeptide may be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted into the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a  
15 recombinant virus that is viable and capable of expressing the polypeptide in infected hosts (e.g., see Logan et al., Proc. Natl. Acad. Sci., USA, 81:3655-3659 (1984)). Alternatively, the vaccinia virus 7.5K promoter may be used (e.g., see, Mackett et al., Proc. Natl. Acad. Sci., USA, 79:7415-7419 (1982); Mackett et al., J. Virol., 49:857-864 (1984); Panicali et al., Proc. Natl. Acad. Sci., USA,  
20 79:4927-4931 (1982)). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver et al., Mol. Cell. Biol., 1:486 (1981)). Shortly after entry of this DNA into target cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid  
25 into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the polypeptide-encoding nucleotide sequence in host cells (Cone et al., Proc.  
30 Natl. Acad. Sci., USA, 81:6349-6353 (1984)). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothioneine IIA promoter and heat shock promoters.



Recently, long-term survival of cytomegalovirus (CMV) promoter versus Rous sarcoma virus (RSV) promoter-driven thymidine kinase (TK) gene therapy in nude mice bearing human ovarian cancer has been studied. Cell killing efficacy of adenovirus-mediated CMV promoter-driven herpes simplex virus TK gene therapy was found to be 2 to 10 time more effective than RSV driven therapy. (Tong et al., 1999, Hybridoma 18(1):93-97). The design of chimeric promoters for gene therapy applications, which call for low level expression followed by inducible high-level expression has also been described. (Suzuki et al., 1996, Human Gene Therapy 7:1883-1893).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter and enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. As mentioned above, the selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., Cell, 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al, Proc. Natl. Acad. Sci., USA, 48:2026 (1962)), and adenine phosphoribosyltransferase (Lowy et al., Cell, 22:817 (1980)) genes, which can be employed in tk-, hgp<sup>r</sup>t- or ap<sup>r</sup>t- cells respectively. Also, antimetabolite resistance-conferring genes can be used as the basis of selection; for example, the genes for dhfr, which confers resistance to methotrexate (Wigler et al., Proc. Natl. Acad. Sci., USA, 77:3567 (1980); O'Hare et al., Proc. Natl. Acad. Sci., USA, 78:1527 (1981); gpt, which confers resistance to mycophenolic acid (Mulligan et al, Proc. Natl. Acad. Sci., USA, 78:2072, (1981)); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al, J. Mol. Biol., 150:1 (1981)); and hyg<sup>r</sup>, which confers resistance to hygromycin (Santerre et al, Gene,

30:147 (1984)). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman et al, Proc. Natl. Acad. Sci., USA, 85:804 (1988)); and ODC (ornithine decarboxylase) which  
5 confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed., (1987)).

The principal vectors contemplated for human gene therapy are derived from retroviral origin. (Wilson, 1997, Clin. Exp. Immunol. 107(Sup. 1):31-32; Bank et al., 1996, Bioessays 18(12):999-1007; Robbins et al., 1998, Pharmacol. Ther. 80(1):35-47). The therapeutic potential of gene transfer and antisense therapy has stimulated the development of many vector systems for treating a variety of tissues. (vasculature, Stephan et al., 1997, Fundam. Clin. Pharmacol. 11(2):97-  
10 110; Feldman et al., 1997, Cardiovasc. Res. 35(3):391-404; Vassalli et al., 1997, Cardiovasc. Res. 35(3):459-69; Baek et al., 1998, Circ. Res. 82(3):295-305; kidney, Lien et al., 1997, Kidney Int. Suppl. 61:S85-8; liver, Ferry et al., 1998, Hum Gene Ther. 9(14):1975-81; muscle, Marshall et al., 1998, Curr. Opin. Genet. Dev. 8(3):360-5). In addition to these tissues, a critical target for human gene  
15 therapy is cancer, either the tumor itself, or associated tissues. (Runnebaum, 1997, Anticancer Res. 17(4B):2887-90; Spear et al., 1998, J. Neurovirol. 4(2):133-47).

Specific examples of viral gene therapy vector systems readily adaptable for use in the methods of the present invention are briefly described below. Retroviral gene delivery has been recently reviewed by Federspiel and Hughes (1998,  
25 Methods in Cell Biol. 52:179-214) which describes in particular, the avian leukosis virus (ALV) retrovirus family (Federspiel et al., Proc. Natl. Acad. Sci., USA, 93:4931 (1996); Federspiel et al., Proc. Natl. Acad. Sci., USA, 91:11241 (1994)). Retroviral vectors, including ALV and murine leukemia virus (MLV) are further described by Svoboda (1998, Gene 206:153-163).

30 Modified retroviral/adenoviral expression systems can be readily adapted for practice of the methods of the present invention. For example, murine leukemia virus (MLV) systems are reviewed by Karavanas et al., 1998, Crit. Rev. in Oncology/Hematology 28:7-30. Adenovirus expression systems are reviewed by

Von Seggern and Nemerow in Gene Expression Systems (ed. Fernandez & Hoeffler, Academic Press, San Diego, CA, 1999, chapter 5, pages 112-157).

- Protein expression systems have been demonstrated to have effective use both in vivo and in vitro. For example, efficient gene transfer to human squamous cell carcinomas by a herpes simplex virus (HSV) type 1 amplicon vector has been described. (Carew et al., 1998, Am. J. Surg. 176:404-408). Herpes simplex virus has been used for gene transfer to the nervous system. (Goins et al., 1997, J. Neurovirol. 3 (Sup. 1):S80-8). Targeted suicide vectors using HSV-TK has been tested on solid tumors. (Smiley et al., 1997, Hum. Gene Ther. 8(8):965-77).
- Herpes simplex virus type 1 vector has been used for cancer gene therapy on colon carcinoma cells. (Yoon et al., 1998, Ann. Surg. 228(3):366-74). Hybrid vectors have been developed to extend the length of time of transfection, including HSV/AAV (adeno-associated virus) hybrids for treating hepatocytes. (Fraefel et al., 1997, Mol. Med. 3(12):813-825).
- Vaccinia virus has been developed for human gene therapy because of its large genome. (Peplinski et al., 1998, Surg. Oncol. Clin. N. Am. 7(3):575-88). Thymidine kinase-deleted vaccinia virus expressing purine nucleoside pyrophosphorylase has been described for use as a tumor directed gene therapy vector. (Puhlman et al., 1999, Human Gene Therapy 10:649-657).
- Adeno-associated virus 2 (AAV) has been described for use in human gene therapy, however AAV requires a helper virus (such as adenovirus or herpes virus) for optimal replication and packaging in mammalian cells. (Snoeck et al., 1997, Exp. Nephrol. 5(6):514-20; Rabinowitz et al., 1998, Curr. Opin. Biotechnol. 9(5):470-5). However, in vitro packaging of an infectious recombinant AAV has been described, making this system much more promising. (Ding et al., 1997, Gene Therapy 4:1167-1172). It has been shown that the AAV mediated transfer of ecotropic retrovirus receptor cDNA allows ecotropic retroviral transduction of established and primary human cells. (Qing et al., 1997, J. Virology 71(7):5663-5667). Cancer gene therapy using an AAV vector expressing human wild-type p53 has been demonstrated. (Qazilbash et al., 1997, Gene Therapy 4:675-682). Gene transfer into vascular cells using AAV vectors has also been shown. (Maeda et al., 1997, Cardiovascular Res. 35:514-521). AAV has been demonstrated as a suitable vector for liver directed gene therapy. (Xiao et al., 1998, J. Virol. 72(12):10222-

6). AAV vectors have been demonstrated for use in gene therapy of brain tissues and the central nervous system. (Chamberlin et al., 1998, Brain Res. 793(1-2):169-75; During et al., 1998, Gene Therapy 5(6):820-7). AAV vectors have also been compared with adenovirus vectors (AdV) for gene therapy of the lung and transfer to human cystic fibrosis epithelial cells. (Teramoto et al., 1998, J. Virol. 72(11):8904-12).

Chimeric AdV/retroviral gene therapy vector systems which incorporate the useful qualities of each virus to create a nonintegrative AdV that is rendered functionally integrative via the intermediate generation of a retroviral producer cell. (Feng et al., 1997, Nat. Biotechnology 15(9):866-70; Bilbao et al., 1997, FASEB J 11(8):624-34). This powerful new generation of gene therapy vector has been adapted for targeted cancer gene therapy. (Bilbao et al., 1998, Adv. Exp. Med. Biol. 451:365-74). Single injection of AdV expressing p53 inhibited growth of subcutaneous tumor nodules of human prostate cancer cells. (Asgari et al., 1997, Int. J. Cancer 71(3):377-82). AdV mediated gene transfer of wild-type p53 in patients with advanced non-small cell lung cancer has been described. (Schuler et al., 1998, Human Gene Therapy 9:2075-2082). This same cancer has been the subject of p53 gene replacement therapy mediated by AdV vectors. (Roth et al., 1998, Semin. Oncol. 25(3 Suppl 8):33-7). AdV mediated gene transfer of p53 inhibits endothelial cell differentiation and angiogenesis in vivo. (Riccioni et al., 1998, Gene Ther. 5(6):747-54). Adenovirus-mediated expression of melanoma antigen gp75 as immunotherapy for metastatic melanoma has also been described. (Hirschowitz et al., 1998, Gene Therapy 5:975-983). AdV facilitates infection of human cells with ecotropic retrovirus and increases efficiency of retroviral infection. (Scott-Taylor, et al., 1998, Gene Ther. 5(5):621-9). AdV vectors have been used for gene transfer to vascular smooth muscle cells (Li et al., 1997, Chin. Med. J.(Engl) 110(12):950-4), squamous cell carcinoma cells (Goebel et al., 1998, Otolaryngol Head Neck Surg 119(4):331-6), esophageal cancer cells (Senmaru et al., 1998, Int J. Cancer 78(3):366-71), mesangial cells (Nahman et al., 1998, J. Investig. Med. 46(5):204-9), glial cells (Chen et al., 1998, Cancer Res. 58(16):3504-7), and to the joints of animals (Ikeda et al., 1998, J. Rheumatol. 25(9):1666-73). More recently, catheter-based pericardial gene transfer mediated by AcV vectors has been demonstrated. (March et al., 1999, Clin. Cardiol. 22(1

Suppl 1):123-9). Manipulation of the AdV system with the proper controlling genetic elements allows for the AdV-mediated regulable target gene expression in vivo. (Burcin et al., 1999, PNAS (USA) 96(2):355-60).

Alphavirus vectors have been developed for human gene therapy applications, with packaging cell lines suitable for transformation with expression cassettes suitable for use with Sindbis virus and Semliki Forest virus-derived vectors. (Polo et al., 1999, Proc. Natl. Acad. Sci., USA, 96:4598-4603). Noncytopathic flavivirus replicon RNA-based systems have also been developed. (Varnavski et al., 1999, Virology 255(2):366-75). Suicide HSV-TK gene containing sinbis virus vectors have been used for cell-specific targeting into tumor cells. (Iijima et al., 1998, Int. J. Cancer 80(1):110-8).

Retroviral vectors based on human foamy virus (HFV) also show promise as gene therapy vectors. (Trobridge et al., 1998, Human Gene Therapy 9:2517-2525). Foamy virus vectors have been designed for suicide gene therapy. (Nestler et al., 1997, Gene Ther. 4(11):1270-7). Recombinant murine cytomegalovirus and promoter systems have also been used as vectors for high level expression. (Manning et al., 1998, J. Virol. Meth. 73(1):31-9; Tong et al., 1998, Hybridoma 18(1):93-7).

Gene delivery into non-dividing cells has been made feasible by the generation of Sendai virus based vectors. (Nakanishi et al., 1998, J. Controlled Release 54(1):61-8).

In other efforts to enable the transformation of non-dividing somatic cells, lentiviral vectors have been explored. Gene therapy of cystic fibrosis using a replication-defective human immunodeficiency virus (HIV) based vector has been described. (Goldman et al., 1997, Human Gene Therapy 8:2261-2268). Sustained expression of genes delivered into liver and muscle by lentiviral vectors has also been shown. (Kafri et al., 1997, Nat. Genet. 17(3):314-7). However, safety concerns are predominant, and improved vector development is proceeding rapidly. (Kim et al., 1998, J. Virol. 72(2):994-1004). Examination of the HIV LTR and Tat yield important information about the organization of the genome for developing vectors. (Sadaie et al., 1998, J. Med. Virol. 54(2):118-28). Thus the genetic requirements for an effective HIV based vector are now better understood. (Gasmi et al., 1999, J. Virol. 73(3):1828-34). Self inactivating vectors, or

conditional packaging cell lines have been described. (for example Zuffery et al., 1998, J. Virol. 72(12):9873-80; Miyoshi et al., 1998, J. Virol. 72(10):8150-7; Dull et al., 1998, J. Virol. 72(11):8463-71; and Kaul et al., 1998, Virology 249(1):167-74). Efficient transduction of human lymphocytes and CD34+ cells by  
5 HIV vectors has been shown. (Douglas et al., 1999, Hum. Gene Ther. 10(6):935-45; Miyoshi et al., 1999, Science 283(5402):682-6). Efficient transduction of nondividing human cells by feline immunodeficiency virus (FIV) lentiviral vectors has been described, which minimizes safety concerns with using HIV based vectors. (Poeschla et al., 1998, Nature Medicine 4(3):354-357). Productive  
10 infection of human blood mononuclear cells by FIV vectors has been shown. (Johnston et al., 1999, J. Virol. 73(3):2491-8).

While many viral vectors are difficult to handle, and capacity for inserted DNA limited, these limitations and disadvantages have been addressed. For example, in addition to simplified viral packaging cell lines, Mini-viral vectors,  
15 derived from human herpes virus, herpes simplex virus type 1 (HSV-1), and Epstein-Barr virus (EBV), have been developed to simplify manipulation of genetic material and generation of viral vectors. (Wang et al., 1996, J. Virology 70(12):8422-8430). Adaptor plasmids have been previously shown to simplify insertion of foreign DNA into helper-independent Retroviral vectors. (1987, J.  
20 Virology 61(10):3004-3012).

Viral vectors are not the only means for effecting gene therapy, as several non-viral vectors have also been described. A targeted non-viral gene delivery vector based on the use of Epidermal Growth Factor/DNA polyplex (EGF/DNA) has been shown to result in efficient and specific gene delivery. (Cristiano, 1998,  
25 Anticancer Res. 18:3241-3246). Gene therapy of the vasculature and CNS have been demonstrated using cationic liposomes. (Yang et al., 1997, J. Neurotrauma 14(5):281-97). Transient gene therapy of pancreatitis has also been accomplished using cationic liposomes. (Denham et al., 1998, Ann. Surg. 227(6):812-20). A chitosan-based vector/DNA complexes for gene delivery have been shown to be  
30 effective. (Erbacher et al., 1998, Pharm. Res. 15(9):1332-9). A non-viral DNA delivery vector based on a terplex system has been described. (Kim et al., 1998, 53(1-3):175-82). Virus particle coated liposome complexes have also been used to

effect gene transfer. (Hirai et al., 1997, Biochem. Biophys. Res. Commun. 241(1):112-8).

5 Cancer gene therapy by direct tumor injections of nonviral T7 vector encoding a thymidine kinase gene has been demonstrated. (Chen et al., 1998, Human Gene Therapy 9:729-736). Plasmid DNA preparation is important for direct injection gene transfer. (Horn et al., 1995, Hum. Gene Ther. 6(5):656-73). Modified plasmid vectors have been adapted specifically for direct injection. (Hartikka et al., 1996, Hum. Gene Ther. 7(10):1205-17).

10 Thus, a wide variety of gene transfer/gene therapy vectors and constructs are known in the art. These vectors are readily adapted for use in the methods of the present invention. By the appropriate manipulation using recombinant DNA/molecular biology techniques to insert an operatively linked src (either active or inactive) into the selected expression/delivery vector, many equivalent vectors for the practice of the present invention can be generated.

15 E. Methods For Modulation of Angiogenesis

The invention provides for a method for the modulation of angiogenesis in a tissue associated with a disease process or condition, and thereby effect events in the tissue which depend upon angiogenesis. Generally, the method comprises administering to the tissue associated with a disease process or condition, a  
20 composition comprising an angiogenesis-modulating amount of a Src protein or nucleic acid vector expressing active or inactive Src.

As described herein, any of a variety of tissues, or organs comprised of organized tissues, can support angiogenesis in disease conditions including skin, muscle, gut, connective tissue, joints, bones and the like tissue in which blood  
25 vessels can invade upon angiogenic stimuli.

The patient treated according to the present invention in its many embodiments is desirably a human patient, although it is to be understood that the principles of the invention indicate that the invention is effective with respect to all mammals, which are intended to be included in the term "patient". In this  
30 context, a mammal is understood to include any mammalian species in which treatment of tissue associated with diseases involving angiogenesis is desirable, particularly agricultural and domestic mammalian species.

Thus the method comprises administering to a patient a therapeutically effective amount of a physiologically tolerable composition containing a Src protein or DNA vector for expressing a Src protein in practicing the methods of the invention.

5       The dosage ranges for the administration of a Src protein depend upon the form of the protein, and its potency, as described further herein. The dosage amounts are large enough to produce the desired effect in which angiogenesis and the disease symptoms mediated by angiogenesis are ameliorated. The dosage should not be so large as to cause adverse side effects, however, such as  
10   hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex of the patient, and extent of the disease in the patient, and can be readily determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

15       A therapeutically effective amount is an amount of Src protein, or nucleic acid encoding for (active or inactive) src protein, sufficient to produce a detectable modulation of angiogenesis in the tissue being treated, ie., an angiogenesis-modulating amount. Modulation of angiogenesis can be measured by CAM assay as described herein, or by other methods known to one skilled in the art.

20       The Src protein or nucleic acid vector expressing the Src protein can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration, and therefore is most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated as  
25   well. Thus, compositions of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

      The therapeutic compositions containing a Src protein or nucleic acid vector expressing the Src protein can be conventionally administered intravenously, as by  
30   injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a



predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

In one preferred embodiment the reagent is administered in a single dosage intravenously. Localized administration can be accomplished by direct injection or  
5 by taking advantage of anatomically isolated compartments, isolating the microcirculation of target organ systems, reperfusion in a circulating system, or catheter based temporary occlusion of target regions of vasculature associated with diseased tissues.

The compositions are administered in a manner compatible with the dosage  
10 formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend  
15 on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations  
20 in the blood in the ranges specified for in vivo therapies are contemplated.

#### 1. Inhibition of Angiogenesis

Inhibition of angiogenesis is important in a variety of diseases, referred to as angiogenic diseases. Such diseases include, but are not limited to, inflammatory disorders such as immune and non-immune inflammation, chronic articular  
25 rheumatism and psoriasis, disorders associated with inappropriate or inopportune invasion of vessels such as diabetic retinopathy, neovascular glaucoma, restenosis, capillary proliferation in atherosclerotic plaques and osteoporosis, and cancer associated disorders, such as solid tumors, solid tumor metastases, angiofibromas, retrolental fibroplasia, hemangiomas, Kaposi sarcoma and the like cancers which  
30 require neovascularization to support tumor growth.

Thus, methods which inhibit angiogenesis in a tissue associated with a disease condition ameliorates symptoms of the disease and, depending upon the disease, can contribute to cure of the disease. In one embodiment, the invention

contemplates inhibition of angiogenesis, per se, in a tissue associated with a disease condition. The extent of angiogenesis in a tissue, and therefore the extent of inhibition achieved by the present methods, can be evaluated by a variety of methods.

5        Thus, in one related embodiment, a tissue to be treated is an inflamed tissue and the angiogenesis to be inhibited is inflamed tissue angiogenesis where there is neovascularization of inflamed tissue. In this class the method contemplates inhibition of angiogenesis in arthritic tissues, such as in a patient with chronic articular rheumatism, in immune or non-immune inflamed tissues, in psoriatic  
10       tissue and the like.

         In another related embodiment, a tissue to be treated is a retinal tissue of a patient with a retinal disease such as diabetic retinopathy, macular degeneration or neovascular glaucoma and the angiogenesis to be inhibited is retinal tissue angiogenesis where there is neovascularization of retinal tissue.

15       In an additional related embodiment, a tissue to be treated is a tumor tissue of a patient with a solid tumor, a metastases, a skin cancer, a breast cancer, a hemangioma or angiofibroma and the like cancer, and the angiogenesis to be inhibited is tumor tissue angiogenesis where there is neovascularization of a tumor tissue. Typical solid tumor tissues treatable by the present methods include lung,  
20       pancreas, breast, colon, laryngeal, ovarian, and the like tissues. Inhibition of tumor tissue angiogenesis is a particularly preferred embodiment because of the important role neovascularization plays in tumor growth. In the absence of neovascularization of tumor tissue, the tumor tissue does not obtain the required nutrients, slows in growth, ceases additional growth, regresses and ultimately  
25       becomes necrotic resulting in killing of the tumor.

         Stated in other words, the present invention provides for a method of inhibiting tumor neovascularization by inhibiting tumor angiogenesis according to the present methods. Similarly, the invention provides a method of inhibiting tumor growth by practicing the angiogenesis-inhibiting methods.

30       The methods are also particularly effective against the formation of metastases because (1) their formation requires vascularization of a primary tumor so that the metastatic cancer cells can exit the primary tumor and (2) their establishment in a secondary site requires neovascularization to support growth of the metastases.

In a related embodiment, the invention contemplates the practice of the method in conjunction with other therapies such as conventional chemotherapy directed against solid tumors and for control of establishment of metastases. The administration of angiogenesis inhibitor is typically conducted during or after  
5 chemotherapy, although it is preferably to inhibit angiogenesis after a regimen of chemotherapy at times where the tumor tissue will be responding to the toxic assault by inducing angiogenesis to recover by the provision of a blood supply and nutrients to the tumor tissue. In addition, it is preferred to administer the angiogenesis inhibition methods after surgery where solid tumors have been  
10 removed as a prophylaxis against metastases.

Insofar as the present methods apply to inhibition of tumor neovascularization, the methods can also apply to inhibition of tumor tissue growth, to inhibition of tumor metastases formation, and to regression of established tumors.

Restenosis is a process of smooth muscle cell (SMC) migration and  
15 proliferation into the tissue at the site of percutaneous transluminal coronary angioplasty which hampers the success of angioplasty. The migration and proliferation of SMC's during restenosis can be considered a process of angiogenesis which is inhibited by the present methods. Therefore, the invention also contemplates inhibition of restenosis by inhibiting angiogenesis according to  
20 the present methods in a patient following angioplasty procedures. For inhibition of restenosis, the inactivated tyrosine kinase is typically administered after the angioplasty procedure because the coronary vessel wall is at risk of restenosis, typically for from about 2 to about 28 days, and more typically for about the first 14 days following the procedure.

25 The present method for inhibiting angiogenesis in a tissue associated with a disease condition, and therefore for also practicing the methods for treatment of angiogenesis-related diseases, comprises contacting a tissue in which angiogenesis is occurring, or is at risk for occurring, with a composition comprising a therapeutically effective amount of an inactivated Src protein or vector expressing  
30 the protein.

Inhibition of angiogenesis and tumor regression occurs as early as 7 days after the initial contacting with the therapeutic composition. Additional or prolonged

exposure to inactive Src protein is preferable for 7 days to 6 weeks, preferably about 14 to 28 days.

## 2. Potentialiation of Angiogenesis

In cases where it is desirable to promote or potentiate angiogenesis, administration of an active Src protein to the tissue is useful. The routes and timing of administration are comparable to the methods described hereinabove for inhibition.

## F. Therapeutic Compositions

The present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with a Src protein or vector capable of expressing a Src protein as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectable either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of

auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of any salt-forming components therein.

- 5 Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as
- 10 isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

- Physiologically tolerable carriers for the active ingredients are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no
- 15 materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

- 20 Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

- A therapeutic composition contains an angiogenesis-modulating amount of an Src protein of the present invention, or sufficient recombinant DNA expression
- 25 vector to express an effective amount of Src protein, typically formulated to contain an amount of at least 0.1 weight percent of Src protein per weight of total therapeutic composition. A weight percent is a ratio by weight of Src protein to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of Src protein per 100 grams of total composition. For DNA expression vectors, the
- 30 amount administered depends on the properties of the expression vector, the tissue to be treated, and the like considerations.

### G. Article of Manufacture

The invention also contemplates an article of manufacture which is a labelled container for providing a Src protein of the invention. An article of manufacture comprises packaging material provided with appropriate labeling for the disease  
5 condition to be treated and a pharmaceutical agent contained within the packaging material.

The pharmaceutical agent in an article of manufacture is any of the compositions of the present invention suitable for providing a Src protein and formulated into a pharmaceutically acceptable form as described herein according  
10 to the disclosed indications. Thus, the composition can comprise a Src protein or a DNA molecule which is capable of expressing a Src protein. The article of manufacture contains an amount of pharmaceutical agent sufficient for use in treating a condition indicated herein, either in unit or multiple dosages.

The packaging material comprises a label which indicates the use of the  
15 pharmaceutical agent contained therein, e.g., for treating conditions assisted by the inhibition or potentiation of angiogenesis, and the like conditions disclosed herein. The label can further include instructions for use and related information as may be required for marketing. The packaging material can include container(s) for storage of the pharmaceutical agent.

20 As used herein, the term packaging material refers to a material such as glass, plastic, paper, foil, and the like capable of holding within fixed means a pharmaceutical agent. Thus, for example, the packaging material can be plastic or glass vials, laminated envelopes and the like containers used to contain a pharmaceutical composition including the pharmaceutical agent.

25 In preferred embodiments, the packaging material includes a label that is a tangible expression describing the contents of the article of manufacture and the use of the pharmaceutical agent contained therein.

### Examples

30 The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed, which would be within

the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

#### 1. Preparation of c-Src Expression Constructs

5 For preparing the expression constructs useful in modulating angiogenesis by the methods of the present invention, c-Src cDNA is manipulated and inserted into an expression construct/vector.

The cDNA sequence encoding for wild-type (i.e., endogenous) chicken c-Src is shown in FIG. 1 (SEQ ID NO.:2) with the encoded amino acid residue sequence shown in FIG. 2 (SEQ ID NO.:3). The encoded protein sequence is translated from the cDNA nucleotide positions 112 to 1713. The nucleic acid sequence corresponding to the nucleic acid sequence of human c-Src cDNA (SEQ ID NO.:4) and encoded amino acid residue (SEQ ID NO.:5) sequences are shown respectively in FIGs. 3 and 4. For the human protein sequence, the coding sequence begins at nucleotide position 134 to 1486 of the cDNA.

15 Wild-type as well as a number of mutated c-Src cDNAs were prepared. Mutated c-Src constructs were prepared by site-directed mutagenesis as described by Kaplan et al., EMBO J., 13:4745-4756 (1994). The mutated c-Src constructs for encoding mutated c-Src proteins for use in the methods of the present invention are described in Kaplan et al., id.. Kaplan et al. describe various mutated c-Src constructs and encoded proteins of useful for the practice of this invention. For example, Kaplan et al. depict several products of chicken c-src alleles in their FIG. 1, including SrcA and Src251.

25 Two categories of c-Src function to modulate angiogenesis are described. As previously discussed, one category contains Src molecules that increase angiogenesis and thus are considered to be active proteins. Wild-type Src along with various mutations are shown in the present invention to induce angiogenesis. One preferred mutation of wild type c-src which functions in this context with respect to its ability to induce blood vessel growth and therefore increase tumor weight in vivo is the Src A mutant having a point mutation at amino acid (aa) residue position 527 changing tyrosine 527 to phenylalanine. This site is normally a site for negative regulation by the c-Src kinase, referred to as kinase CSK. 30 When CSK phosphorylates aa527 in the wild-type src, the protein is inactivated.

However, in mutated Src A, the regulatory tyrosine converted to phenylalanine thus conferring upon the protein a constitutively (i.e., permanently) active protein not subject to inactivation by phosphorylation.

5 Mutations in src have also been shown to have the opposite modulatory effect on angiogenesis, inhibiting angiogenesis instead of stimulating it. Such mutations are referred to as inactive src mutations. Proteins having mutation that confer this inhibitory activity are also referred to as dominant negative Src proteins in that they inhibit neovascularization, including that resulting from endogenous activity of Src as well as enhanced Src activity resulting from growth factor stimulation.  
10 Thus certain mutations of wild type c-src of the present invention can also function as a dominant negative with respect to their ability to block blood vessel growth, and for example, therefore decrease tumor weight in vivo.

Such preferred inhibitory c-Src protein includes the Src 251 in which only the first 251 amino acids of Src are expressed. This construct lacks the entire kinase  
15 domain and is therefore referred to as "kinase dead" src protein. A second construct is the Src (K295M) mutation in which the lysine amino acid residue 295 is mutated into a methionine. This point mutation in the kinase domain prevents ATP binding and also blocks kinase-dependent Src functions related to vascular cell and tumor cell signaling and proliferation.

20 For example, for the mutation at residue 527, as long as the resultant mutated amino acid residue is not tyrosine, serine, or threonine, the present invention contemplates that the presence of an alternate amino acid at the desired position will result in a Src protein with a desired active, angiogenesis promoting modulatory activity.

25 With respect to the point mutations, any mutation resulting in the desired inhibitory or stimulatory activity is contemplated for use in this invention. Fusion protein constructs combining the desired src protein (mutation or fragment thereof) with expressed amino acid tags, antigenic epitopes, fluorescent protein, or other such protein or peptides are also contemplated, so long as the desired modulating  
30 effect of the src protein is intact.



**TABLE I**

| <u>Src/Mutation</u> | <u>Src Function</u> | <u>Effect on Angiogenesis</u> |
|---------------------|---------------------|-------------------------------|
| c-Src               | +                   | active stimulates             |
| SrcA (T527F)        | +                   | active stimulates             |
| 5 Src527(point)     | +                   | active stimulates             |
| Src251              | -                   | inactive inhibits             |
| Src (truncate)      | -                   | inactive inhibits             |
| Src(K295M)          | -                   | inactive inhibits             |
| Src295 (point)      | -                   | inactive inhibits             |

10

One preferred expression construct for use in the present invention is the RCASBP(A) construct (SEQ ID NO.:1). This expression vector is based on a series of replication competent avian sarcoma viruses with an enhanced Bryan polymerase (BP) for improved titre, and is specific for the A type envelope glycoprotein expressed on normal avian cells (Reviewed in Methods in Cell Biology, 52:179-214 (1997); see also, Hughes et al., 1987, J. Virol. 61:3004-3012; Fekete & Cepko, 1993, Mol. Cellular Biol. 13(4):2604-2613; Itoh et al., 1996, Development 122:291-300; and Stott et al., 1998, BioTechniques 24:660-666). The complete sequence of RCASBP(A) (SEQ ID NO.:1) is given in the attached sequence listing, and a restriction map of the construct is depicted as FIG. 10, referred to herein as RCAS.

The original Src 251 construct was subcloned by Dr. Pam Schwartzberg, at NIH in Dr. Harold Varmus' laboratory. Briefly, cloning of a src cDNA sequence for expression thereof was accomplished by inserting a linker containing Not I-BstB1-Not I restriction sites into a unique Not I site in the 5' end of Src 251. Src has a unique Cla I site at the 3' end. Digestion of Src 251 with BstB1 and Cla I generated a BstB1-ClaI fragment which was then ligated into the Cla I site on RCASBP(A). A BstB1 overhang allows for ligation with a Cla I overhang that will not be recut with Cla I. The src constructs suitable for use in practicing the present invention are readily obtained in the above vector by first digesting the RCAS vector containing Src 251 with Not I and Cla I (in a DAM+ background) to allow for insertion of a similarly digested Src cDNA. Therefore this initial RCASBP(A) construct containing Src 251 was further used to subclone all other

30

Src constructs as described above and in Kaplan et al. (1994, The EMBO J. 13(20):4745-4756), into RCASBP(A) via a Not I-Cla I fragment generated through the Src 251 construction. To produce the desired c-src mutations in the cDNA, standard site-directed mutagenesis procedures familiar to one of ordinary skill in the art were utilized. PCR primers designed to incorporate the desired mutations were also designed with restriction sites to facilitate subsequent cloning steps. Entire segments of Src encoding nucleic acid sequences are deleted from the nucleic acid constructs through PCR amplification techniques based on the known cDNA sequences of chicken, human and the like homologs of Src and subsequent formation of new constructs.

In one embodiment of the invention, the 3' PCR primer used to amplify src nucleic acids also encodes for an in-frame sequence. Use of this primer adds a 9E10-myc epitope tag to the carboxyl terminus of the subsequent Src construct.

The following amino acids were added after amino acid 251 of Src to generate vector constructs containing the 9E10-myc epitope tag: VDMEQKLIAEEDLN (SEQ ID NO.: 6). Two separate PCRs were carried out for each construct and similar results were obtained. All mutant constructs constructed by PCR were also sequenced by PCR to confirm predicted DNA sequence of clones. Wild-type and mutated Src cDNAs for use in the expression systems of the present invention are also available from Upstate Biotech Laboratories, Lake Placid, NY which sells avian as well as human src, and several kinase dead and activated mutated forms.

Alternative expression vectors for use in the expressing the Src proteins of the present invention also include adenoviral vectors as described in US Patent Numbers 4,797,368, 5,173,414, 5,436,146, 5,589,377, and 5,670,488. Alternative methods for the delivery of the Src modulatory proteins include delivery of the Src cDNA with a non-viral vector system as described in US Patent Number 5,675,954 and delivery of the cDNA itself as naked DNA as described in US Patent Number 5,589,466. Delivery of constructs of this invention is also not limited to topical application of a viral vector as described in the CAM assay system below. For example, viral vector preparations are also injected intravenously for systemic delivery into the vascular bed. These vectors are also targetable to sites of increased neovascularization by localized injection of a tumor, as an example.

In vitro expressed proteins are also contemplated for delivery thereof following expression and purification of the selected Src protein by methods useful for delivery of proteins or polypeptides. One such method includes liposome delivery systems, such as described in US Patent Numbers 4,356,167, 5,580,575, 5,542,935 and 5,643,599. Other vector and protein delivery systems are well known to those of ordinary skill in the art for use in the expression and/or delivery of the Src proteins of the present invention.

2. Characterization of the Untreated Chick Chorioallantoic Membrane (CAM)

A. Preparation of the CAM

Angiogenesis can be induced on the chick chorioallantoic membrane (CAM) after normal embryonic angiogenesis has resulted in the formation of mature blood vessels. Angiogenesis has been shown to be induced in response to specific cytokines or tumor fragments as described by Leibovich et al., Nature, 329:630 (1987) and Ausprunk et al., Am. J. Pathol., 79:597 (1975). CAMs were prepared from chick embryos for subsequent induction of angiogenesis and inhibition thereof. Ten day old chick embryos were obtained from McIntyre Poultry (Lakeside, CA) and incubated at 37°C with 60% humidity. A small hole was made through the shell at the end of the egg directly over the air sac with the use of a small crafts drill (Dremel, Division of Emerson Electric Co. Racine WI). A second hole was drilled on the broad side of the egg in a region devoid of embryonic blood vessels determined previously by candling the egg. Negative pressure was applied to the original hole, which resulted in the CAM (chorioallantoic membrane) pulling away from the shell membrane and creating a false air sac over the CAM. A 1.0 centimeter (cm) x 1.0 cm square window was cut through the shell over the dropped CAM with the use of a small model grinding wheel (Dremel). The small window allowed direct access to the underlying CAM.

The resultant CAM preparation was then either used at 6 days of embryogenesis, a stage marked by active neovascularization, without additional treatment to the CAM reflecting the model used for evaluating effects on embryonic neovascularization or used at 10 days of embryogenesis where angiogenesis has subsided. The latter preparation was thus used in this invention

for inducing renewed angiogenesis in response to cytokine treatment or tumor contact as described below.

### 3. CAM Angiogenesis Assay

#### A. Angiogenesis Induced by Growth Factors

5 Angiogenesis has been shown to be induced by cytokines or growth factors.

Angiogenesis was induced by placing a 5 millimeter (mm) X 5 mm Whatman filter disk (Whatman Filter paper No.1) saturated with Hanks Balanced Salt Solution (HBSS, GIBCO, Grand Island, NY) or HBSS containing 2 micrograms/milliliter ( $\mu\text{g/ml}$ ) recombinant basic fibroblast growth factor (bFGF) or vascular endothelial cell growth factor (VEGF) (Genzyme, Cambridge, MA) on the CAM of either a 9 or 10 day chick embryo in a region devoid of blood vessels and the windows were latter sealed with tape. Other concentrations of growth factors are also effective at inducing blood vessel growth. For assays where inhibition of angiogenesis is evaluated with intravenous injections of antagonists, angiogenesis is first induced with 1-2  $\mu\text{g/ml}$  bFGF or VEGF in fibroblast growth medium. Angiogenesis was monitored by photomicroscopy after 72 hours.

#### B. Embryonic Angiogenesis

The CAM preparation for evaluating the effect of angiogenesis inhibitors on the natural formation of embryonic neovasculature is the 6 day embryonic chick embryo as previously described. At this stage in development, the blood vessels are undergoing de novo growth and thus provides a useful system for assessing angiogenesis modulation by the Src proteins of the present invention. The CAM system is prepared as described above with the exception that the assay is performed at embryonic day 6 rather than at day 9 or 10.

### 25 4. Modulation of Angiogenesis as Measured in the CAM Assay

To assess the effect of Src proteins on angiogenesis, the following assays were performed on 10 day old chick CAM preparations. Five  $\mu\text{g}$  of RCAS constructs prepared as described in Example 1 were transfected into the chicken immortalized fibroblast line, DF-1 (gift of Doug Foster, U. of Minn.). This cell line as well as primary chick embryo fibroblasts were capable of producing virus, however the DF-1 cell line produced higher titres. Viral supernatants were collected from subconfluent DF-1 producer cell lines in serum free CLM media [composition: F-10 media base supplemented with DMSO, folic acid, glutamic acid, and MEM

vitamin solution]. Thirty-five ml of viral supernatant were concentrated by ultracentrifugation at 4°C for 2 hours at 22,000 rpm. These concentrated viral pellets were resuspended in 1/100 the original volume in serum-free CLM media, aliquoted and stored at -80°C. The titre was assessed by serial dilution of a control viral vector having a nucleotide sequence encoding green fluorescent protein (GFP), referred to as RCAS-GFP, infection on primary chick embryo fibroblasts that were incubated for 48-72 hours. The titres of viral stock that were obtained following concentration routinely exceeded 10<sup>8</sup> I.u./ml. For the CAM assay using the viral stocks, cortisone acetate soaked Whatman filter disks 6 mm in diameter were prepared in 3 mg/ml cortisone acetate for 30 minutes in 95% ethanol. The disks were dried in a laminar flow hood and then soaked on 20 µl of viral stock per disk for 10 minutes. These disks were applied to the CAM of 9 or 10 day chick embryos and sealed with cellophane tape and incubated at 37°C for 18-24 hr. Then either mock PBS or growth factors were added at a concentration of 5 µg/ml to the CAM in a 20 µl volume of the appropriate virus stock as an additional boost of virus to the CAM tissue. After 72 hours, the CAMs were harvested and examined for changes in the angiogenic index as determined by double blind counting of the number of branch points in the CAM underlying the disk. For kinase assays, the tissue underlying the disk was harvested in RIPA, homogenized with a motorized grinder and Src immunoprecipitated from equivalent amounts of total protein and subjected to an *in vitro* kinase assay using a FAK-GST fusion protein as a substrate. For the immunofluorescence studies, CAM tissue underlying the disks were frozen in OCT, a cryopreservative, sectioned at 4 µm, fixed in acetone for 1 minute, incubated in 3% normal goat serum for 1 hour, followed by an incubation in primary rabbit anti-phosphorylated ERK antibody as described previously (Eliceiri et al., *J. Cell Biol.*, 140:1255-1263 (1998), washed in PBS and detected with a fluorescent secondary antibody.

A. Activation of Endogenous Src by bFGF or VEGF

To assess the effects of growth factors on Src activity in modulating angiogenesis, the following assays were performed. Tissue extracts of 10 day old chick CAMs that had been exposed to bFGF or VEGF (2 µg/ml) for 2 hours were lysed. Endogenous Src was immunoprecipitated from equivalent amounts of total protein and subjected to an *in vitro* immune complex kinase assay using a FAK-

GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose.

The results of the assay are shown in FIG. 5 where the increase in Src activity is evident in the increased density of the gel with either bFGF or VEGF treatment as compared to untreated (mock) samples that are indicative of baseline Src activity in the CAM assay. Both bFGF and VEGF resulted in approximately a 2 fold increase of endogenous Src activity present in the CAM. The above kinase assay blot was also probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

10        B.     Effect of Retrovirus-Mediated Gene Expression of Src A on Angiogenesis in the Chick CAM

The following assay was performed to assess the effect of mutated Src proteins on angiogenesis in the CAM preparation. For the assay, 9 day old chick CAMs were exposed to RCAS-Src A or RCAS-GFP expressing retroviruses or buffer for 72 hour following the protocol described above.

The results of this assay are shown in FIG. 6A where the level of angiogenesis was quantified as described above. Representative photomicrographs (4x) were taken with a stereomicroscope as shown in FIG. 6B. Baseline endogenous Src activity has an angiogenic index of approximately 50. In contrast, CAMs treated with retroviral vector-expressed RCAS-Src A having a point mutation at amino acid residue position 527 from a tyrosine to a phenylalanine resulted in an enhancement (induction) of angiogenesis of an angiogenic index of approximately 90. The enhancement of Src-A mediated angiogenesis is also evident in the photographs shown in FIG. 6B.

25        C.     Retroviral Expression of Src A Activates Vascular MAP Kinase Phosphorylation

The effect of Src A as compared to growth factors VEGF and PMA on vascular MAP kinase phosphorylation was also assessed following the assay procedures described above and herein. Tissue extracts of 10 day old chick CAMs exposed to VEGF or PMA (another mitogen at a comparable concentration) for 30 minutes were compared to those infected with Src A-expressing retrovirus for 48 hours. Src was then immunoprecipitated from equivalent amounts of total protein extract and subjected to an in vitro immune complex kinase assay using a

FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose.

The results of this assay are shown in FIG. 7A where untreated CAMs (NT) exhibit base-line endogenous Src-mediated vascular MAP kinase phosphorylation.

5 Both VEGF and PMA resulted in an approximate 2 fold increase over baseline. In contrast, Src A enhanced the activity approximately 5 to 10 fold over that seen with untreated samples.

Aliquots of the above whole tissue lysates were also measured for endogenous ERK phosphorylation by immunoblotting with an anti-phospho-ERK antibody as shown in FIG. 7B. For this assessment, 10 day old CAMs were infected with either mock RCAS or RCAS that expresses SRC A. After two days, CAMs were dissected, cryopreserved in OCT and sectioned at 4  $\mu$ m. Sections were immunostained with an anti-phosphorylated ERK antibody (New England Biolabs), washed and detected with a goat anti-rabbit FITC-conjugated secondary antibody. Fluorescent images were captured on a cooled-CCD camera (Princeton Inst.). The photomicrographs indicate enhanced immunofluorescence with Src A-treated preparations compared to mock controls.

D. Selective Requirement for Src Activity During VEGF, but Not bFGF-Induced Angiogenesis

20 To assess the effect of Src modulatory activity on growth factor induced angiogenesis, the following assays were performed. Nine day old chick CAMs were exposed to the retroviral vector preparation that expressed the dominant negative Src mutation referred to as Src 251 or Src K295M as previously described. RCAS-Src 251 or control RCAS-GFP retroviruses or buffer CAMS were treated for 20 hours and then incubated for an additional 72 hours in the presence or absence of bFGF or VEGF.

The level of angiogenesis, quantified as described above, is shown in FIG. 8A. Representative photomicrographs (6x), shown in FIG. 8B, were taken with a stereomicroscope. FIG. 8C illustrates a blot probed with an anti-Src antibody to confirm the expression of Src 251 in transfected cells as compared to mock treatments.

The results of the assays described above indicate that both bFGF and VEGF treated CAMS in the presence of RCAS-GFP controls induced angiogenesis over

the Src-mediated baseline angiogenesis seen with mock or untreated CAM preparations. The expressed dominant negative mutant Src 251 was effective at inhibiting VEGF-induced angiogenesis back to baseline levels while not effective at inhibiting bFGF-mediated angiogenesis. The photomicrographs shown in FIG. 8B pictorially confirm the data shown in FIG. 8A. Thus, retrovirally expressed Src 251 is an effective angiogenesis inhibitor, when angiogenesis is induced with VEGF.

Applications of the Src proteins of this invention with other angiogenesis models as described in the Examples below are contemplated in the present invention.

5     5. Regression of Tumor Tissue Growth With Src Modulators as Measured by In Vivo Rabbit Eye Model Assay

The effect of Src modulators on growth factor-induced angiogenesis can be observed in naturally transparent structures as exemplified by the cornea of the eye. New blood vessels grow from the rim of the cornea, which has a rich blood supply, toward the center of the cornea, which normally does not have a blood supply. Stimulators of angiogenesis, such as bFGF, when applied to the cornea induce the growth of new blood vessels from the rim of the cornea. Antagonists of angiogenesis, applied to the cornea, inhibit the growth of new blood vessels from the rim of the cornea. Thus, the cornea undergoes angiogenesis through an invasion of endothelial cells from the rim of the cornea into the tough collagen-packed corneal tissue which is easily visible. The rabbit eye model assay therefore provides an *in vivo* model for the direct observation of stimulation and inhibition of angiogenesis following the implantation of compounds directly into the cornea of the eye.

25     A.     In Vivo Rabbit Eye Model Assay

1)     Angiogenesis Induced by Growth Factors

Angiogenesis is induced in the *in vivo* rabbit eye model assay with growth factors bFGF or VEGF and is described in the following sections.

30     a.     Preparation of Hydron Pellets Containing Growth Factor and Monoclonal Antibodies

Hydron polymer pellets containing growth factor are prepared as described by D'Amato, et al., Proc. Natl. Acad. Sci., USA, 91:4082-



4085 (1994). The individual pellets contain 650 ng of the growth factors separately bound to sucralfate (Carafet, Marion Merrell Dow Corporation) to stabilize the growth factor and ensure its slow release into the surrounding tissue. In addition, hydron pellets are prepared containing a desired Src-expressing

5 retrovirus as previously described. The pellets are cast in specially prepared Teflon pegs that have a 2.5 mm core drilled into their surfaces. Approximately 12 ul of casting material is placed into each peg and polymerized overnight in a sterile hood. Pellets are then sterilized by ultraviolet irradiation. Effects of Src proteins are then assessed as previously described.

10 6. In Vivo Regression of Tumor Tissue Growth With Src Modulators As Measured by Chimeric Mouse:Human Assay

An in vivo chimeric mouse:human model is generated by replacing a portion of skin from a SCID mouse with human neonatal foreskin. The in vivo chimeric mouse:human model is prepared essentially as described in Yan, et al., J. Clin. Invest., 91:986-996 (1993). Briefly, a 2 cm<sup>2</sup> square area of skin is surgically

15 removed from a SCID mouse (6-8 weeks of age) and replaced with a human foreskin. The mouse is anesthetized and the hair removed from a 5 cm<sup>2</sup> area on each side of the lateral abdominal region by shaving. Two circular graft beds of 2 cm<sup>2</sup> are prepared by removing the full thickness of skin down to the fascia. Full

20 thickness human skin grafts of the same size derived from human neonatal foreskin are placed onto the wound beds and sutured into place. The graft is covered with a Band-Aid which is sutured to the skin. Micropore cloth tape is also applied to cover the wound.

The M21-L human melanoma cell line or MDA 23.1 breast carcinoma cell

25 line (ATCC HTB 26;  $\alpha_v\beta_3$  negative by immunoreactivity of tissue sections with mAb LM609), are used to form the solid human tumors on the human skin grafts on the SCID mice. A single cell suspension of  $5 \times 10^6$  M21-L or MDA 23.1 cells is injected intradermally into the human skin graft. The mice are then observed for 2 to 4 weeks to allow growth of measurable human tumors.

30 After a measurable tumor is established, retrovirus preparations of the present invention or PBS is injected into the mouse tail vein. Following a 2-3 week period, the tumor is excised and analyzed by weight and histology. The effect of expressed Src proteins of the present invention on the tumors is then assessed.

7. In Vitro Regression of Human Tumor Tissue Growth With Src Modulators As Measured by CAM Assay

5 Tumor growth depends on angiogenesis (Folkman, 1992; Weidner et al., 1991; Brooks et al., 1994b). In fact, recent reports suggest that tumor growth is susceptible to the anti-angiogenic effects of VEGF receptor antagonists (Kim et al., 1993). Therefore, we examined whether suppression of angiogenesis by delivery of kinase-deleted Src 251 would influence the growth of a human medulloblastoma (DAOY), a highly angiogenic tumor known to produce VEGF and very little bFGF (data not shown).

10 The 3 and 6 day DAOY medulloblastoma tumor growth assays were performed in the chick CAM essentially as previously described (Brooks et al., 1994).  $5 \times 10^6$  DAOY cells cultured in RPMI 1640 containing 10% fetal calf serum were washed and seeded on the CAM of a 10 day embryo to produce DAOY tumor fragments. After 7 days 50 mg tumor fragments were dissected and  
15 reseeded on another 10 day embryo and incubated for another 3 or 6 days with the topical application (25 $\mu$ l) of either control RCAS-GFP retrovirus, RCAS-Src 251, or mock treatment. Using the whole tissue confocal imaging of infected tumors as a guide we were able to determine that there was significant expression of the RCAS constructs around and within the tumor fragment with this topical approach.  
20 Tumor resections and weighing were performed in a double blind manner removing only the easily definable solid tumor mass (Brooks et al., 1994). The wet tumor weights after 3 or 6 days were compared with initial weight and the percent change of tumor weight determined for each group.

25 These tumors readily grow on the CAM and produces active angiogenesis (FIG. 9) allowing us to selectively target the avian-derived tumor vasculature by using an avian-specific RCAS retrovirus.

FIG. 9 depicts results that show retroviral delivery of RCAS-Src 251 to human tumors growing on the chick CAM reverses tumor growth. FIG. 9A shows human medulloblastomas that were grown on the CAM of chick embryos as described above. Retrovirus containing RCAS-GFP or RCAS-Src 251 was  
30 topically applied to preestablished tumors of greater than 50 mg. A representative micrograph of a medulloblastoma tumor fragment infected with RCAS-GFP expressing GFP reveals exclusive expression in the tumor blood vessels

(arrowhead) as detected by optical sectioning with a Bio Rad laser confocal scanning microscope (bar=500 $\mu$ m). FIG. 9B shows results from tumors treated as above that were allowed to grow for 3 or 6 days after which they were resected and wet weights determined. Data are expressed as the mean change in tumor weight (from the 50 mg tumor starting weight) +/- SEM of 2 replicates. RCAS-Src 251 had a significant impact on tumor growth after 3 days (\*,  $P < 0.002$ ) and 6 days (\*\*,  $P < 0.05$ ). FIG. 9C shows representative stereomicrographs of medulloblastoma tumors surgically removed from the embryos were taken with an Olympus stereomicroscope (bar=350 $\mu$ m). (Lower panel) A high magnification micrograph of each tumor showing the vasculature of each tumor in detail (bar=350 $\mu$ m). The arrowhead indicates blood vessel disruption in RCAS-Src251-treated tumors.

The results show that delivery of RCAS containing Src 251 to preestablished medulloblastomas resulted in selective viral expression in the tumor-associated blood vessels (FIG. 9A) and this ultimately led to the regression of these tumors within the span of six days (FIG. 9B). Importantly, the tumor-associated blood vessels in animals treated with virus containing Src 251 were severely disrupted and fewer in number compared to the tumor vessels in control animals (FIG. 9C). The fact that RCAS-GFP infected tumors showed GFP localization only in the tumor vasculature suggests that the anti-tumor effects observed with retrovirally delivered Src 251 were due to its anti-angiogenic properties.

The foregoing examples and the accompanying description are illustrative, and are not to be taken as limiting. The present invention also is not to be limited in scope by the cell line deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention. Any cell line that is functionally equivalent is within the scope of this invention. The deposit of material does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

## What Is Claimed Is:

1. An article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of modulating angiogenesis in a tissue associated with a disease condition, wherein said packaging material comprises a label which indicates that said pharmaceutical composition can be used for treating disease conditions by modulating angiogenesis, and wherein said pharmaceutical composition comprises a Src protein or an oligonucleotide having a nucleotide sequence capable of expressing said protein.
2. The article of manufacture of claim 1 wherein said Src protein is an active Src protein and said modulating potentiates angiogenesis.
3. The article of manufacture of claim 2 wherein said active Src protein is Src A.
4. The article of manufacture of claim 2 wherein said tissue has poor circulation.
5. The article of manufacture of claim 1 wherein said tyrosine kinase Src protein is an inactive Src protein and said modulating inhibits angiogenesis.
6. The article of manufacture of claim 5 wherein said inactive Src protein is Src 251 or Src K295M.
7. The article of manufacture of claim 5 wherein said tissue is inflamed and said condition is arthritis or rheumatoid arthritis.
8. The article of manufacture of claim 5 wherein said tissue is a solid tumor or solid tumor metastasis.
9. The article of manufacture of claim 8 wherein said administering is conducted in conjunction with chemotherapy.
10. The article of manufacture of claim 5 wherein said tissue is retinal tissue and said condition is retinopathy, diabetic retinopathy or macular degeneration.
11. The article of manufacture of claim 5 wherein said tissue is at the site of coronary angioplasty and said condition is restenosis.
12. The article of manufacture of claim 1 wherein said administering comprises intravenous, transdermal, intrasynovial, intramuscular, or oral administration.

13. The article of manufacture of claim 1 wherein said administering comprises a single dose intravenously.

14. The article of manufacture of claim 1 wherein said pharmaceutical composition further comprises a liposome.

5 15. The article of manufacture of claim 1 wherein said pharmaceutical composition comprises a viral expression vector capable of expressing said nucleotide sequence.

16. The article of manufacture of claim 1 wherein said pharmaceutical composition comprises an non-viral expression vector capable of expressing said  
10 nucleotide sequence.

17. A method for modulating angiogenesis in a tissue associated with a disease condition comprising administering to said tissue a pharmaceutical composition comprising a Src protein or a nucleotide sequence capable of expressing said protein.

18. The method of claim 17 wherein said Src protein is an active Src protein and said modulating potentiates angiogenesis.

19. The method of claim 18 wherein said active Src protein is Src A.

20. The method of claim 18 wherein said tissue has poor circulation.

21. The method of claim 17 wherein said Src protein is an inactive Src  
20 protein and said modulating inhibits angiogenesis.

22. The method of claim 21 wherein said inactive Src protein is Src 251 or Src K295M.

23. The method of claim 21 wherein said tissue is inflamed and said condition is arthritis or rheumatoid arthritis.

25 24. The method of claim 21 wherein said tissue is a solid tumor or solid tumor metastasis.

25. The method of claim 24 wherein said administering is conducted in conjunction with chemotherapy.

26. The method of claim 21 wherein said tissue is retinal tissue and said  
30 condition is retinopathy, diabetic retinopathy or macular degeneration.

27. The method of claim 21 wherein said tissue is at the site of coronary angioplasty and said tissue is at risk for restenosis.

28. The method of claim 17 wherein said administering comprises intravenous, transdermal, intrasynovial, intramuscular, or oral administration.

29. The method of claim 17 wherein said administering comprises a single dose intravenously.

5 30. The method of claim 17 wherein said pharmaceutical composition further comprises a liposome.

31. The method of claim 17 wherein said pharmaceutical composition comprises an retroviral expression vector capable of expressing said nucleotide sequence.

10 32. The method of claim 17 wherein said pharmaceutical composition comprises an non-viral expression vector capable of expressing said nucleotide sequence.

33. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a viral gene transfer vector containing a nucleic acid  
15 and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein, said src protein having any amino acid residue at codon 527 except for tyrosine, serine or threonine.

34. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a non-viral gene transfer vector containing a nucleic  
20 acid and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein and said src protein having any amino acid residue at codon 527 except tyrosine, serine or threonine.

35. A pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a viral gene transfer vector containing a nucleic acid  
25 and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein having no kinase activity.

36. A pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a non-viral gene transfer vector containing a nucleic  
30 acid and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein, said src protein having no kinase activity.

37. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a therapeutic amount of a src protein in a

pharmaceutically acceptable carrier or excipient; said src protein having any amino acid residue at codon 527 except tyrosine, serine or threonine.

38. A pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a a src protein in a pharmaceutically acceptable carrier or excipient; said src protein having no kinase activity.
- 5

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(21) International Application Number:</b> PCT/US99/11780<br><br><b>(22) International Filing Date:</b> 28 May 1999 (28.05.99)<br><br><b>(30) Priority Data:</b><br>60/087,220                      29 May 1998 (29.05.98)                      US<br><br><b>(71) Applicant (for all designated States except US):</b> THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US).<br><br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> CHERESH, David, A. [US/US]; 327 Via Andalusia, Encinitas, CA 92024 (US). ELICEIRI, Brian [US/US]; 935 Laguna #5, Carlsbad, CA 92008 (US). SCHWARTZBERG, Pamela, L. [US/US]; 49/4A38, National Humane Genome Research Institute, National Institute of Health, Bethesda, MD 20892-4472 (US).<br><br><b>(74) Agents:</b> CEPURITIS, Talivaldis et al.; Olson & Hierl, Ltd., 36th floor, 20 North Wacker Drive, Chicago, IL 60606 (US). |           | <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF ANGIOGENESIS USING TYROSINE KINASE SRC   |           |  |
| <b>(57) Abstract</b><br><br>The present invention describes methods for modulating angiogenesis in tissues using Src protein, modified Src protein, and nucleic acids encoding for such. Particularly the invention describes methods for inhibiting angiogenesis using an inactive Src protein, or nucleic acids encoding therefor, or for potentiating angiogenesis using an active Src protein, or nucleic acids encoding therefor. The invention also describes the use of gene delivery systems for providing nucleic acids encoding for the Src protein, or modified forms thereof.   |           |  |



## CHICKEN c-SRC cDNA

(SEQ ID NO:2)

1 tctgacaccc atctgtctgt ctgtctgtgt gctgcaggag ctgagctgac tctgtctgtg  
61 cctcgcgtac cactgtggcc aggcggtagc tgggacgtgc agcccaccac catggggagc  
121 agcaagagca agcccaagga ccccgccag cgccggcgca gcctggagcc acccgacagc  
181 acccaccacg ggggattccc agcctcgcag accccaaca agacagcagc ccccgacacg  
241 caccgcaccc ccagccgctc ctttgggacc gtggccaccg agccaagct ctccgggggc  
301 ttcaaacatt ctgacaccgt tacgtgccg cagcgtgccg gggcactggc tggcggcgctc  
361 accactttcg tggctctcta cgactacgag tcccggactg aaacggactt gtcctcaag  
421 aaaggagaac gctgcagat tgtaacaac acggaagggtg actggtggct ggctcattcc  
481 ctactacag gacagacggg ctacatcccc agtaactatg tcgcgccctc agactccatc  
541 caggctgaag agtggtaact tgggaagatc actcgtcggg agtcgagcgc gctgtctctc  
601 aaccccgaaa acccccgggg aacctcttg gtccgggaga gcgagacgac aaaaggtgcc  
661 tattgctct cgtttctga cttgacaac gccaggggc tcaatgtgaa gcactacaag  
721 atccgcaagc tggacagcgg cggttctac atcacctcac gcacacagtt cagcagcctg  
781 cagcagctgg tggcctacta ctccaaacat gctgatggct tgtgccaccg cctgaccaac  
841 gtctgcccca cgtccaagcc ccagaccag ggactcgcca aggacgcgtg ggaaatcccc  
901 cgaggagtcgc tgcggctgga ggtgaagctg gggcagggct gctttggaga ggtctggatg  
961 gggacctgga acggcaccac cagagtggcc ataaagactc tgaagcccg gaccatgtcc  
1021 ccggaggcct tcctgcagga agccaagtg atgaagaagc tccggcatga gaagctgggt  
1081 cagctgtacg cagtgggtgc ggaagagccc atctacatcg tctctgagta catgagcaag  
1141 gggagcctcc tggatttctt gaaggagag atgggcaagt acctgcggct gccacagctc  
1201 gtcgatatgg ctgctcagat tgcatccggc atggcctatg tggagaggat gaactacgtg  
1261 caccgagacc tgcgggcggc caacatcctg gtgggggaga acctggtgtg caaggtggct  
1321 gactttgggc tggcacgcct catcaggagc aacaggtaca cagcacggca aggtgccaag  
1381 ttcccatca agtggacagc ccccgaggca gccctctatg gccgggtcac catcaagtcg  
1441 gatgtctggt ccttcggcat cctgtgact gagctgacca ccaagggccg ggtgccatac  
1501 ccagggatgg tcaacaggga ggtgctggac caggtggaga ggggctaccg catgccctgc  
1561 ccgcccagat gcccagatc gctgcatgac ctcatgtgcc agtgcctggc gagggaccct  
1621 gaggagcggc ccactttga gtacctgag gccttctgg aggactacti cacctcgaca  
1681 gagccccagt accagcctgg agagaacct taggcctgga gtcctctcg gaccagaggc  
1741 ctgcgtgtgg ggtacaggg

FIG. 1

## CHICKEN cSRC ENCODED PROTEIN

(SEQ ID NO:3)

MGSSKSKPKDPSQRRRSLEPPDSTHHGGFPASQTPNKTA  
PDTHRTSPRSFGTVATEPKLFGGFNTSDTVTSPQRAGALA  
GGVTTFVALYDYESTETDLSFKKGERLQIVNNTSGDWL  
AHSLTTGQTGYIPSNYVAPSDSIQAEWYFGKITRRESER  
LLNPENPRGTFLVRESETTKGAYCLSVSDFDNAKGLNVK  
HYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHR  
LTNVCPTSKPQTQGLAKDAWEIPRESLRLEVKLGGGCFGE  
VWMGTWNGTTRVAIKTLKPGTMSPEAFLQEAQVMKKLRHE  
KLVQLYAVVSEEPYIVTEYMSKGSLLDFLKGEMGKYLRL  
PQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENL  
VCKVADFGRLARLIEDNEYTARQGAKFPIKWTAPAAALYGR  
FTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLDQVERG  
YRMPCPPECPESLHDLMCQCWRRDPEERPTFEYLQAFLE  
DYFTSTEPQYQPGENL

FIG. 2

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## HUMAN c-SRC cDNA

(SEQ ID NO:4)

1 gcgccgcgtc ccgcaggccg tgatgccgc cgcgcggagg tggcccggac cgcagtgcc  
61 caagagagct ctaatggtac caagtacag gttggcttta ctgtgactcg gggacgccag  
121 agctcctgag aagatgtcag caatacaggc cgctggcca tccggtacag aatgtattgc  
181 caagtacaac tccacggca ctgccgagca ggacctgccc ttctgcaaag gagacgtgct  
241 caccattgtg gccgtcacca aggaccccaa ctggtacaaa gccaaaaaca aggtgggccc  
301 tgagggcatc atcccagcca actacgtcca gaagcgggag ggctgaagg cgggtacca  
361 actcagcctc atgccttggt tccacggcaa gatcacacgg gagcaggctg agcggcttct  
421 gtaccgccc gagacaggcc tgttctggt gcgggagagc accaactacc ccggagacta  
481 cacgtgtgc gtgagctgcg acggcaagg gtgagcactac cgcatcatgt accatgccag  
541 caagctcagc atcgacgagg aggtgtactt tgagaacctc atgcagctgg tggagcacta  
601 cacctcagac gcagatggac tctgtacgc cctcattaaa ccaaagggtca tggagggcac  
661 agtggcggcc caggatgagt tctaccgcag cggctgggccc ctgaacatga aggagctgaa  
721 gctgctgcag accatcggga agggggaggt cggagacgtg atgctgggcg attaccgagg  
781 gaacaaagtc gccgtcaagt gcattaagaa cgacgccact gccaggcct tcttggtga  
841 agcctcagtc atgacgcaac tgcggcatag caacctgggt cagctcctgg gcgtgatcgt  
901 ggaggagaag ggcgggctct acatcgtcac tgagtacatg gccaggggga gccttgtgga  
961 ctacctgcgg tctaggggtc ggtcagtgtt gggcggagac tgtctctca agttctcgt  
1021 agatgtctgc gaggccatgg aatacctgga gggcaacaat ttcgtgcac gagacctggc  
1081 tgcccgaat gtgtggtgt ctgaggacaa cgtggccaag gtcagcgact ttggtctcac  
1141 caaggaggcg tccagcacc aggacacggg caagctgcca gtcaagtga cagcccctga  
1201 ggcctgaga gagaagaaat tctcactaa gtctgacgtg tggagtctc gaatccttct  
1261 ctgggaaatc tactccttg ggcgagtgc ttatcaaga attcccctga aggacgtcgt  
1321 cctcgggtg gagaagggt acaagatgga tgccccgcac ggctgcccgc ccgcagtcta  
1381 tgaagtcag aagaactgtt ggcacctgga cgcgcctatg cggccctcct tctacagct  
1441 ccgagagcag cttagcaca tcaaaccca cgagctgcac ctgtgacggc tggcctccgc  
1501 ctgggtcatg ggctgtggg gactgaacct ggaagatcat ggacctggtg cccctgctca  
1561 ctggggccga gcctgaactg agccccagcg ggctggcggg ccttttct gcgtccagc  
1621 ctgcaccct ccggccccgt ctctctgga cccacctgtg gggcctgggg agccactga  
1681 ggggccaggg aggaaggagg ccacggagcg ggaggcagcg cccaccacg tggggttcc  
1741 ctggcctccc gccactgcc ttcttagagt ttattcctt tctttttt agattttt  
1801 tccgtgtgtt ttttttat tttttcaa gataaggaga aagaaagta ccagcaaat  
1861 ggcattttac aagaagtac aatctattt ttctgtctt gccgtgagg gtggggggga  
1921 ccgggcccc ctctagggac cctcgcctc agcctcatt cccattctgt gtccatgtc  
1981 ccgtgtctcc tcggtcgccc cgtgttgcc ctgacctat ttgactgtt tgcatgcgc  
2041 cgaggcagac gtctgtcagg ggcttggtt tctgtgccc ctgccaccg cccaccgcg  
2101 ttgtgagatg gaattgtaat aaaccacgcc atgaggacac cgcgccccgc ctggcgctt  
2161 cctccaccga aaaaaaaaa aaaaaaa

FIG. 3

4 / 10

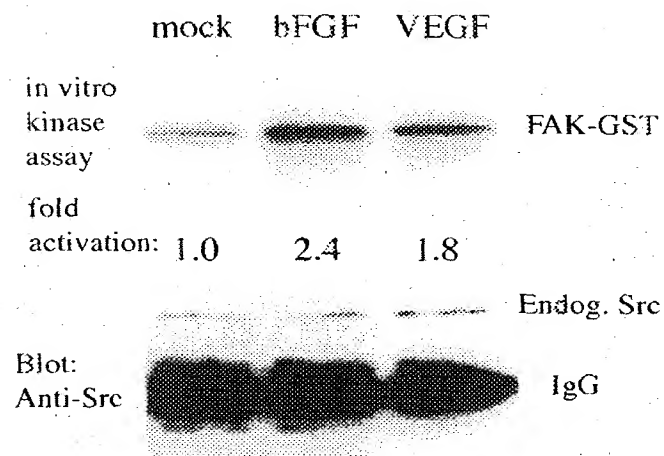
## HUMAN c-SRC ENCODED PROTEIN

(SEQ ID NO:5)

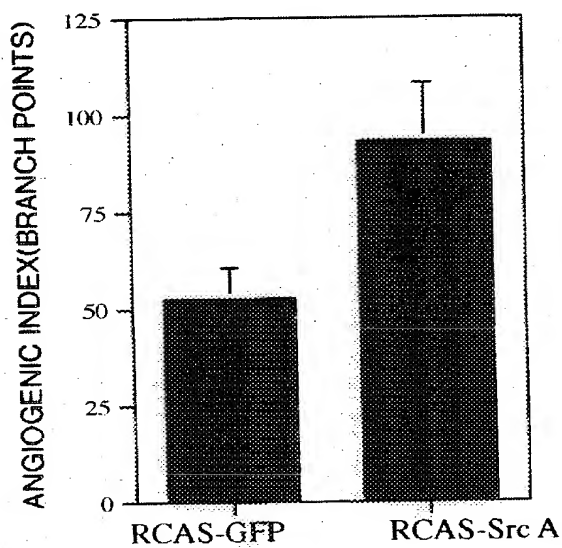
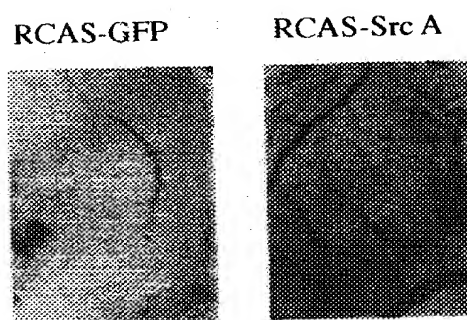
MSAIQAAWPSGTECIAKYNFHGTAEQDLPFCKGDVLTIVAVTKD  
PNWYKAKNKVGREGIIPANYVQKREGVKAGTKLSLMPWFHGKIT  
REQAERLLYPPETGLFLVRESTNYPGDYTLCVSCDGKVEHYRIMY  
HASKLSIDEEVYFENLMQLVEHYTSDADGLCTRLIKPKVMEGTVA  
AQDEFYRSGWALNMKELKLLQTIGKGEGDVMLGDYRGNKVAV  
KCIKNATAQAFLAEASVMTQLRHSNLVQLLGVIVEEKGGLYIVTE  
YMAKGSLVDYLRSRGRSVLGGDCLLKFSLDVCEAMEYLEGNFVH  
RDLAARNVLVSEDNVAKVSDFGLTKEASSTQDTGKLPVKWTAPEAL  
REKKFSTKSDVWSFGILLWEIYSFGRVPYPRIPLKD VVPRVEKGYKM  
DAPDGCPPAVYEVMKNCWHLDAAMRPSFLQLREQLEHIKTHELHL

FIG. 4

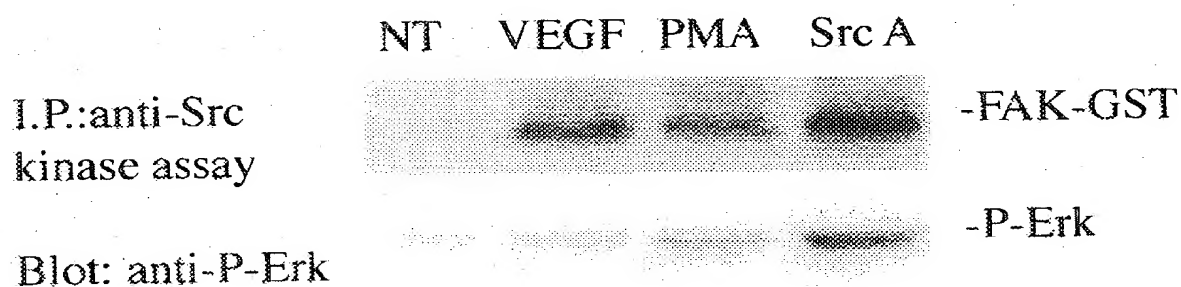
### Activation of endogenous Src activity by bFGF and VEGF

**FIG. 5**

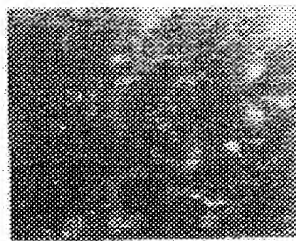
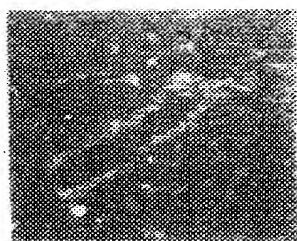
Effect of RCAS-mediated expression of  
Src A on angiogenesis in the chick CAM

**FIG. 6A****FIG. 6B**

## Retroviral expression of Src A activates vascular MAP kinase phosphorylation



Mock



Src A

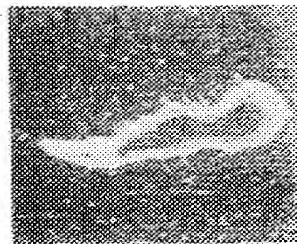
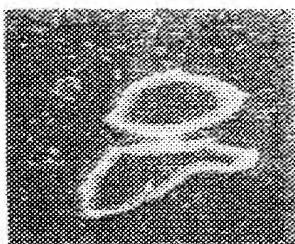


FIG. 7

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# Selective requirement for Src activity during VEGF, but not bFGF-induced angiogenesis

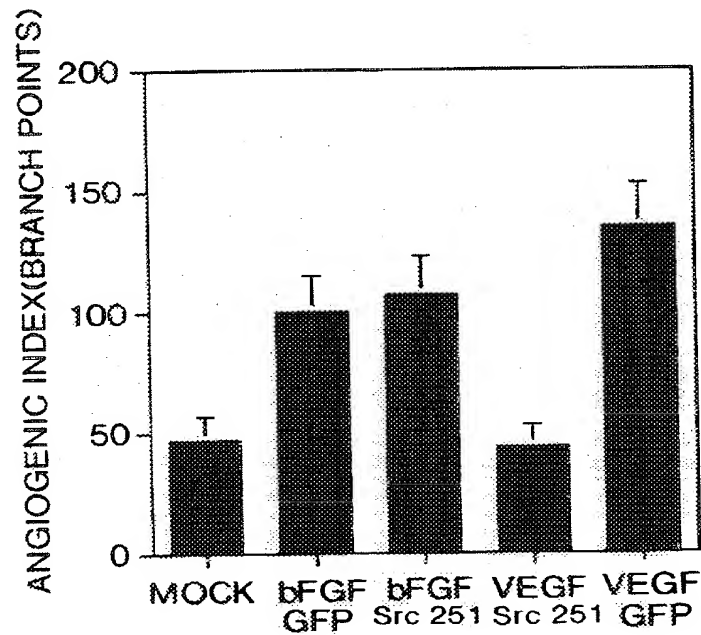


FIG. 8A

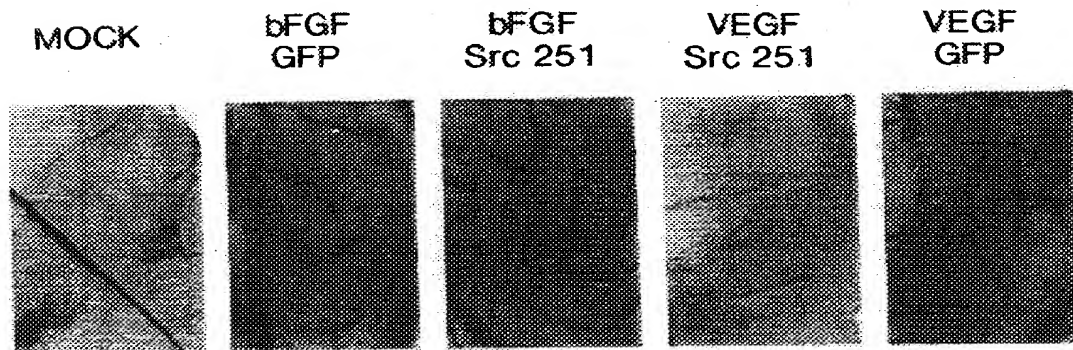
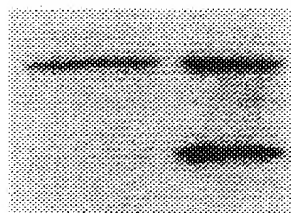


FIG. 8B

MOCK Src 251-myc

BLOT:ANTI-Src



- Src 251

FIG. 8C



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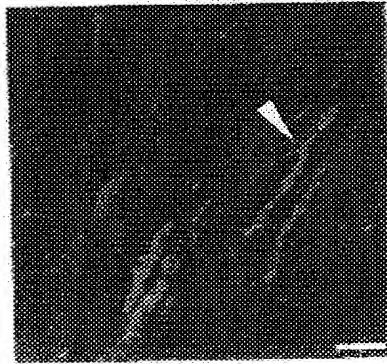


FIG. 9A

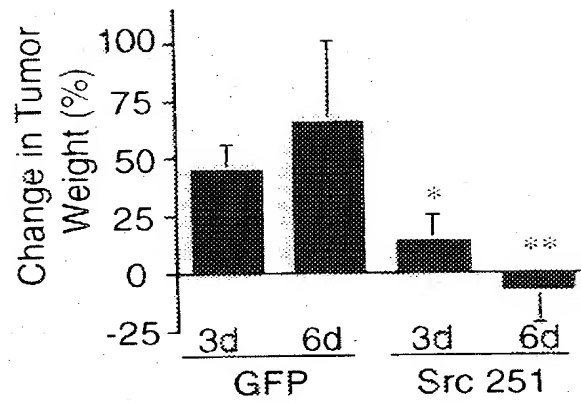


FIG. 9B

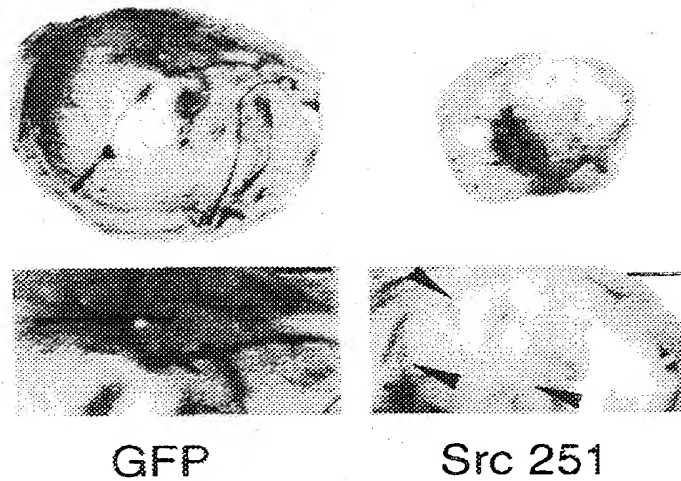


FIG. 9C

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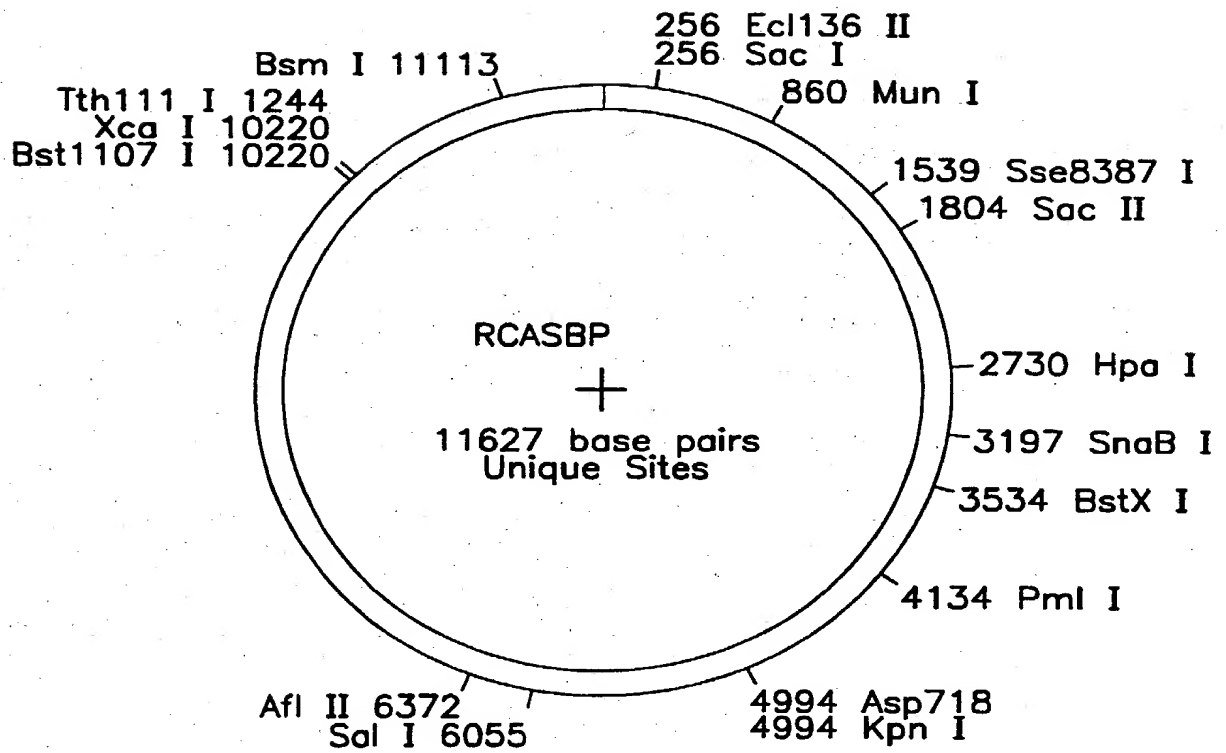



FIG. 10

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|  |                          |             |
|--|--------------------------|-------------|
| <b>DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)</b><br><br><input type="checkbox"/> Declaration Submitted with Initial Filing      OR <input type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required) | Attorney Docket Number   | TSRI 651.1  |
|  | First Named Inventor     | Cheresh     |
|  | <b>COMPLETE IF KNOWN</b> |             |
|  | Application Number       | /           |
|  | Filing Date              | 28 MAY 1999 |
|  | Group Art Unit           |             |
|  | Examiner Name            |             |

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Methods and Compositions Useful for Modulation of Angiogenesis  
 Using Tyrosine Kinase Src

the specification of which

(Title of the Invention)

☐ is attached hereto  
 OR

☒ was filed on (MM/DD/YYYY) 05/28/1999 as United States Application Number or PCT International

Application Number PCT/US99/11780 and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

| Prior Foreign Application Number(s) | Country | Foreign Filing Date (MM/DD/YYYY) | Priority Not Claimed     | Certified Copy Attached? |                          |
|-------------------------------------|---------|----------------------------------|--------------------------|--------------------------|--------------------------|
|                                     |         |                                  |                          | YES                      | NO                       |
|                                     |         |                                  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|                                     |         |                                  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|                                     |         |                                  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|                                     |         |                                  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

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|-----------------------|--------------------------|--|
| 60/087,220            | 05/29/1998               | <input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto. |

(Page 1 of 2)

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| U.S. Parent Application or PCT Parent Number | Parent Filing Date (MM/DD/YYYY) | Parent Patent Number (if applicable) |
|--|---------------------------------|--------------------------------------|
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| Dolores T. Kenney | 31,269              | Talivaldis Cepuritis  | 20,818              |
| Seymour Rothstein | 19,369              | Daniel J. Deneufbourg | 33,675              |

☒ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.


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| City    | <u>CHICAGO</u>                           | State     | <u>IL</u>             |
| Country | <u>US</u>                                | Telephone | <u>(312) 580-1180</u> |
|         |  | Fax       | <u>(312) 580-1189</u> |

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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| Given Name (first and middle (if any)) |                          | Family Name or Surname  |                |
| <u>David A.</u>                        |                          | <u>Cheresh</u>  |                |
| Inventor's Signature                   | <u>David A. Cheresh</u>  |   | Date           |
|  |                          |   | <u>6/22/99</u> |
| Residence: City                        | <u>Encinitas</u>         | State   | <u>CA</u>      |
|  |                          | Country   | <u>US</u>      |
| Post Office Address                    | <u>327 Via Andalusia</u> |   |                |
| Post Office Address                    |                          |   |                |
| City                                   | <u>Encinitas</u>         | State   | <u>CA</u>      |
|  |                          | ZIP   | <u>92024</u>   |
|  |                          | Country   | <u>US</u>      |

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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**DECLARATION****ADDITIONAL INVENTOR(S)**  
**Supplemental Sheet**  
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|   |                                |       |           |   |              |             |           |
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| Given Name (first and middle [if any])            |                                |       |           | Family Name or Surname  |              |             |           |
| 2-00 <u>Brian</u>                                 |                                |       |           | <u>Eliceiri</u>   |              |             |           |
| Inventor's Signature                              | <u>Brian</u>                   |       |           | <u>Eliceiri</u>   |              | Date        | 6/22/99   |
| Residence: City                                   | <u>Carlsbad</u>                | State | <u>CA</u> | Country   | <u>US</u>    | Citizenship | <u>US</u> |
| Post Office Address                               | <u>3104 Hataca Road</u>        |       |           |   |              |             |           |
| Post Office Address                               |                                |       |           |   |              |             |           |
| City  | <u>Carlsbad</u>                | State | <u>CA</u> | ZIP   | <u>92009</u> | Country     | <u>US</u> |
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| Given Name (first and middle [if any])            |                                |       |           | Family Name or Surname  |              |             |           |
| 3-00 <u>Pamela L.</u>                             |                                |       |           | <u>Schwartzberg</u>   |              |             |           |
| Inventor's Signature                              | <u>Pamela L. Schwartzberg</u>  |       |           |   |              | Date        | 7-6-99    |
| Residence: City                                   | <u>Bethesda</u>                | State | <u>MD</u> | Country   | <u>US</u>    | Citizenship | <u>US</u> |
| Post Office Address                               | <u>5521 Spruce Tree Avenue</u> |       |           |   |              |             |           |
| Post Office Address                               |                                |       |           |   |              |             |           |
| City  | <u>Bethesda</u>                | State | <u>MD</u> | ZIP   | <u>20814</u> | Country     | <u>US</u> |
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|   |                                |       |           |   |              |             |           |
| Inventor's Signature                              |                                |       |           |   |              | Date        |           |
| Residence: City                                   |                                | State |           | Country   |              | Citizenship |           |
| Post Office Address                               |                                |       |           |   |              |             |           |
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| Timothy L. Harney  | <del>38,174</del>   |   |                     |
| Mark Chao          | <del>37,293</del>   |   |                     |
| Joseph M. Kuo      | <del>38,943</del>   |   |                     |
| James C. Haight    | <del>25,588</del>   |   |                     |
| David R. Sadowski  | <del>32,808</del>   |   |                     |
| Robert Benson      | <del>33,612</del>   |   |                     |
| Jack Spiegel       | <del>34,477</del>   |   |                     |
| Susan S. Rucker    | <del>35,762</del>   |   |                     |
| Steven M. Ferguson | <del>38,448</del>   |   |                     |
| Stephen L. Finley  | <del>36,357</del>   |   |                     |
| John Peter Kim     | <del>38,514</del>   |   |                     |

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09/701500

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## SEQUENCE LISTING

<110> CHERESH, David A.  
ELICEIRI, Brian  
SCHWARTZBERG, Pamela L.

<120> METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF  
ANGIOGENESIS USING TYROSINE KINASE SRC

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<140> US 09/701,500  
<141> 2000-11-29

<150> PCT/US99/11780  
<151> 1999-05-28

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 Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp  
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 Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp  
 195 200 205  
 Ser Gly Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln  
 210 215 220  
 Gln Leu Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg  
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 Leu Thr Asn Val Cys Pro Thr Ser Lys Pro Gln Thr Gln Gly Leu Ala  
 245 250 255  
 Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys  
 260 265 270  
 Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly  
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 Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro  
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 Glu Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Glu  
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 Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile  
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Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly  
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 Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His  
 370 375 380  
 Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys  
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 Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr  
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 Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu  
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 Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe  
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 Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro  
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 Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg  
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 Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp Leu Met Cys  
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 Gln Cys Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu  
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                   Met Ser Ala Ile Gln Ala Ala Trp Pro Ser Gly Thr  
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 gaa tgt att gcc aag tac aac ttc cac ggc act gcc gag cag gac ctg 217  
 Glu Cys Ile Ala Lys Tyr Asn Phe His Gly Thr Ala Glu Gln Asp Leu  
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 Pro Asn Trp Tyr Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile  
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 cca gcc aac tac gtc cag aag cgg gag ggc gtg aag gcg ggt acc aaa 361  
 Pro Ala Asn Tyr Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys  
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 ctc agc ctc atg cct tgg ttc cac ggc aag atc aca cgg gag cag gct 409  
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 gag cgg ctt ctg tac ccg ccg gag aca ggc ctg ttc ctg gtg cgg gag 457  
 Glu Arg Leu Leu Tyr Pro Pro Glu Thr Gly Leu Phe Leu Val Arg Glu  
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 Ser Thr Asn Tyr Pro Gly Asp Tyr Thr Leu Cys Val Ser Cys Asp Gly  
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 Lys Val Glu His Tyr Arg Ile Met Tyr His Ala Ser Lys Leu Ser Ile  
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 gac gag gag gtg tac ttt gag aac ctc atg cag ctg gtg gag cac tac 601  
 Asp Glu Glu Val Tyr Phe Glu Asn Leu Met Gln Leu Val Glu His Tyr  
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 acc tca gac gca gat gga ctc tgt acg cgc ctc att aaa cca aag gtc 649  
 Thr Ser Asp Ala Asp Gly Leu Cys Thr Arg Leu Ile Lys Pro Lys Val  
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 atg gag ggc aca gtg gcg gcc cag gat gag ttc tac cgc agc ggc tgg 697  
 Met Glu Gly Thr Val Ala Ala Gln Asp Glu Phe Tyr Arg Ser Gly Trp  
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 gcc ctg aac atg aag gag ctg aag ctg ctg cag acc atc ggg aag ggg 745  
 Ala Leu Asn Met Lys Glu Leu Lys Leu Leu Gln Thr Ile Gly Lys Gly  
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| gag ttc gga gac gtg atg ctg ggc gat tac cga ggg aac aaa gtc gcc | 793  |
| Glu Phe Gly Asp Val Met Leu Gly Asp Tyr Arg Gly Asn Lys Val Ala |      |
| 205 210 215 220   |      |
| gtc aag tgc att aag aac gac gcc act gcc cag gcc ttc ctg gct gaa | 841  |
| Val Lys Cys Ile Lys Asn Asp Ala Thr Ala Gln Ala Phe Leu Ala Glu |      |
| 225 230 235   |      |
| gcc tca gtc atg acg caa ctg cgg cat agc aac ctg gtg cag ctc ctg | 889  |
| Ala Ser Val Met Thr Gln Leu Arg His Ser Asn Leu Val Gln Leu Leu |      |
| 240 245 250   |      |
| ggc gtg atc gtg gag gag aag ggc ggg ctc tac atc gtc act gag tac | 937  |
| Gly Val Ile Val Glu Glu Lys Gly Gly Leu Tyr Ile Val Thr Glu Tyr |      |
| 255 260 265   |      |
| atg gcc aag ggg agc ctt gtg gac tac ctg cgg tct agg ggt cgg tca | 985  |
| Met Ala Lys Gly Ser Leu Val Asp Tyr Leu Arg Ser Arg Gly Arg Ser |      |
| 270 275 280   |      |
| gtg ctg ggc gga gac tgt ctc ctc aag ttc tcg cta gat gtc tgc gag | 1033 |
| Val Leu Gly Gly Asp Cys Leu Leu Lys Phe Ser Leu Asp Val Cys Glu |      |
| 285 290 295 300   |      |
| gcc atg gaa tac ctg gag ggc aac aat ttc gtg cat cga gac ctg gct | 1081 |
| Ala Met Glu Tyr Leu Glu Gly Asn Asn Phe Val His Arg Asp Leu Ala |      |
| 305 310 315   |      |
| gcc cgc aat gtg ctg gtg tct gag gac aac gtg gcc aag gtc agc gac | 1129 |
| Ala Arg Asn Val Leu Val Ser Glu Asp Asn Val Ala Lys Val Ser Asp |      |
| 320 325 330   |      |
| ttt ggt ctc acc aag gag gcg tcc agc acc cag gac acg ggc aag ctg | 1177 |
| Phe Gly Leu Thr Lys Glu Ala Ser Ser Thr Gln Asp Thr Gly Lys Leu |      |
| 335 340 345   |      |
| cca gtc aag tgg aca gcc cct gag gcc ctg aga gag aag aaa ttc tcc | 1225 |
| Pro Val Lys Trp Thr Ala Pro Glu Ala Leu Arg Glu Lys Lys Phe Ser |      |
| 350 355 360   |      |
| act aag tct gac gtg tgg agt ttc gga atc ctt ctc tgg gaa atc tac | 1273 |
| Thr Lys Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile Tyr |      |
| 365 370 375 380   |      |
| tcc ttt ggg cga gtg cct tat cca aga att ccc ctg aag gac gtc gtc | 1321 |
| Ser Phe Gly Arg Val Pro Tyr Pro Arg Ile Pro Leu Lys Asp Val Val |      |
| 385 390 395   |      |
| cct cgg gtg gag aag ggc tac aag atg gat gcc ccc gac ggc tgc ccg | 1369 |
| Pro Arg Val Glu Lys Gly Tyr Lys Met Asp Ala Pro Asp Gly Cys Pro |      |
| 400 405 410   |      |
| ccc gca gtc tat gaa gtc atg aag aac tgc tgg cac ctg gac gcc gcc | 1417 |
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Gly Asp Val Leu Thr Ile Val Ala Val Thr Lys Asp Pro Asn Trp Tyr  
          35                          40                  45  
  
Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile Pro Ala Asn Tyr  
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Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys Leu Ser Leu Met  
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|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Pro | Trp | Phe | His | Gly | Lys | Ile | Thr | Arg | Glu | Gln | Ala | Glu | Arg | Leu | Leu | 85  | 90  | 95  |
| Tyr | Pro | Pro | Glu | Thr | Gly | Leu | Phe | Leu | Val | Arg | Glu | Ser | Thr | Asn | Tyr | 100 | 105 | 110 |
| Pro | Gly | Asp | Tyr | Thr | Leu | Cys | Val | Ser | Cys | Asp | Gly | Lys | Val | Glu | His | 115 | 120 | 125 |
| Tyr | Arg | Ile | Met | Tyr | His | Ala | Ser | Lys | Leu | Ser | Ile | Asp | Glu | Glu | Val | 130 | 135 | 140 |
| Tyr | Phe | Glu | Asn | Leu | Met | Gln | Leu | Val | Glu | His | Tyr | Thr | Ser | Asp | Ala | 145 | 150 | 155 |
| Asp | Gly | Leu | Cys | Thr | Arg | Leu | Ile | Lys | Pro | Lys | Val | Met | Glu | Gly | Thr | 165 | 170 | 175 |
| Val | Ala | Ala | Gln | Asp | Glu | Phe | Tyr | Arg | Ser | Gly | Trp | Ala | Leu | Asn | Met | 180 | 185 | 190 |
| Lys | Glu | Leu | Lys | Leu | Leu | Gln | Thr | Ile | Gly | Lys | Gly | Glu | Phe | Gly | Asp | 195 | 200 | 205 |
| Val | Met | Leu | Gly | Asp | Tyr | Arg | Gly | Asn | Lys | Val | Ala | Val | Lys | Cys | Ile | 210 | 215 | 220 |
| Lys | Asn | Asp | Ala | Thr | Ala | Gln | Ala | Phe | Leu | Ala | Glu | Ala | Ser | Val | Met | 225 | 230 | 235 |
| Thr | Gln | Leu | Arg | His | Ser | Asn | Leu | Val | Gln | Leu | Leu | Gly | Val | Ile | Val | 245 | 250 | 255 |
| Glu | Glu | Lys | Gly | Gly | Leu | Tyr | Ile | Val | Thr | Glu | Tyr | Met | Ala | Lys | Gly | 260 | 265 | 270 |
| Ser | Leu | Val | Asp | Tyr | Leu | Arg | Ser | Arg | Gly | Arg | Ser | Val | Leu | Gly | Gly | 275 | 280 | 285 |
| Asp | Cys | Leu | Leu | Lys | Phe | Ser | Leu | Asp | Val | Cys | Glu | Ala | Met | Glu | Tyr | 290 | 295 | 300 |
| Leu | Glu | Gly | Asn | Asn | Phe | Val | His | Arg | Asp | Leu | Ala | Ala | Arg | Asn | Val | 305 | 310 | 315 |
| Leu | Val | Ser | Glu | Asp | Asn | Val | Ala | Lys | Val | Ser | Asp | Phe | Gly | Leu | Thr | 325 | 330 | 335 |
| Lys | Glu | Ala | Ser | Ser | Thr | Gln | Asp | Thr | Gly | Lys | Leu | Pro | Val | Lys | Trp | 340 | 345 | 350 |
| Thr | Ala | Pro | Glu | Ala | Leu | Arg | Glu | Lys | Lys | Phe | Ser | Thr | Lys | Ser | Asp | 355 | 360 | 365 |
| Val | Trp | Ser | Phe | Gly | Ile | Leu | Leu | Trp | Glu | Ile | Tyr | Ser | Phe | Gly | Arg | 370 | 375 | 380 |

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Val Pro Tyr Pro Arg Ile Pro Leu Lys Asp Val Val Pro Arg Val Glu  
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Lys Gly Tyr Lys Met Asp Ala Pro Asp Gly Cys Pro Pro Ala Val Tyr  
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Glu Val Met Lys Asn Cys Trp His Leu Asp Ala Ala Met Arg Pro Ser  
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His Leu  
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## SEQUENCE LISTING

<110> The Scripps Research Institute et al.

<120> METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF  
ANGIOGENESIS USING TYROSINE KINASE SRC

<130> TSRI 651.1

<140> Not yet known

<141> To be determined

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<151> 1998-05-29

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&lt;222&gt; (112) .. (1710)

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cctcgcgtac cactgtggcc aggcggtagc tgggacgtgc agccaccac c atg ggg 117

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Met Gly  
1

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| Ser Ser Lys Ser Lys Pro Lys Asp Pro Ser Gln Arg Arg Arg Ser Leu |     |
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| gag cca ccc gac agc acc cac cac ggg gga ttc cca gcc tcg cag acc | 213 |
| Glu Pro Pro Asp Ser Thr His His Gly Gly Phe Pro Ala Ser Gln Thr |     |
| 20 25 30  |     |
| ccc aac aag aca gca gcc ccc gac acg cac cgc acc ccc agc cgc tcc | 261 |
| Pro Asn Lys Thr Ala Ala Pro Asp Thr His Arg Thr Pro Ser Arg Ser |     |
| 35 40 45 50   |     |
| ttt ggg acc gtg gcc acc gag ccc aag ctc ttc ggg ggc ttc aac act | 309 |
| Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe Asn Thr |     |
| 55 60 65  |     |
| tct gac acc gtt acg tcg ccg cag cgt gcc ggg gca ctg gct ggc ggc | 357 |
| Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala Gly Gly |     |
| 70 75 80  |     |
| gtc acc act ttc gtg gct ctc tac gac tac gag tcc cgg act gaa acg | 405 |
| Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr Glu Thr |     |
| 85 90 95  |     |
| gac ttg tcc ttc aag aaa gga gaa cgc ctg cag att gtc aac aac acg | 453 |
| Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn Asn Thr |     |
| 100 105 110   |     |
| gaa ggt gac tgg tgg ctg gct cat tcc ctc act aca gga cag acg ggc | 501 |
| Glu Gly Asp Trp Trp Leu Ala His Ser Leu Thr Thr Gly Gln Thr Gly |     |
| 115 120 125 130   |     |
| tac atc ccc agt aac tat gtc gcg ccc tca gac tcc atc cag gct gaa | 549 |
| Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln Ala Glu |     |
| 135 140 145   |     |
| gag tgg tac ttt ggg aag atc act cgt cgg gag tcc gag cgg ctg ctg | 597 |
| Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg Leu Leu |     |
| 150 155 160   |     |
| ctc aac ccc gaa aac ccc cgg gga acc ttc ttg gtc cgg gag agc gag | 645 |
| Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu Ser Glu |     |
| 165 170 175   |     |
| acg aca aaa ggt gcc tat tgc ctc tcc gtt tct gac ttt gac aac gcc | 693 |
| Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp Asn Ala |     |
| 180 185 190   |     |
| aag ggg ctc aat gtg aag cac tac aag atc cgc aag ctg gac agc ggc | 741 |
| Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp Ser Gly |     |
| 195 200 205 210   |     |
| ggc ttc tac atc acc tca cgc aca cag ttc agc agc ctg cag cag ctg | 789 |
| Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln Gln Leu |     |
| 215 220 225   |     |
| gtg gcc tac tac tcc aaa cat gct gat ggc ttg tgc cac cgc ctg acc | 837 |
| Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg Leu Thr |     |
| 230 235 240   |     |
| aac gtc tgc ccc acg tcc aag ccc cag acc cag gga ctc gcc aag gac | 885 |

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| Asn | Val | Cys | Pro | Thr | Ser | Lys | Pro | Gln | Thr | Gln | Gly | Leu | Ala | Lys | Asp |      |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| 245 |     |     |     |     |     |     | 250 |     |     |     |     | 255 |     |     |     |      |
| gcg | tgg | gaa | atc | ccc | cgg | gag | tcg | ctg | cgg | ctg | gag | gtg | aag | ctg | ggg | 933  |
| Ala | Trp | Glu | Ile | Pro | Arg | Glu | Ser | Leu | Arg | Leu | Glu | Val | Lys | Leu | Gly |      |
| 260 |     |     |     |     |     | 265 |     |     |     |     | 270 |     |     |     |     |      |
| cag | ggc | tgc | ttt | gga | gag | gtc | tgg | atg | ggg | acc | tgg | aac | ggc | acc | acc | 981  |
| Gln | Gly | Cys | Phe | Gly | Glu | Val | Trp | Met | Gly | Thr | Trp | Asn | Gly | Thr | Thr |      |
| 275 |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |     | 290 |      |
| aga | gtg | gcc | ata | aag | act | ctg | aag | ccc | ggc | acc | atg | tcc | ccg | gag | gcc | 1029 |
| Arg | Val | Ala | Ile | Lys | Thr | Leu | Lys | Pro | Gly | Thr | Met | Ser | Pro | Glu | Ala |      |
|     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     | 305 |     |      |
| ttc | ctg | cag | gaa | gcc | caa | gtg | atg | aag | aag | ctc | cgg | cat | gag | aag | ctg | 1077 |
| Phe | Leu | Gln | Glu | Ala | Gln | Val | Met | Lys | Lys | Leu | Arg | His | Glu | Lys | Leu |      |
|     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |     |     |      |
| gtt | cag | ctg | tac | gca | gtg | gtg | tcg | gaa | gag | ccc | atc | tac | atc | gtc | act | 1125 |
| Val | Gln | Leu | Tyr | Ala | Val | Val | Ser | Glu | Glu | Pro | Ile | Tyr | Ile | Val | Thr |      |
|     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |     |     |      |
| gag | tac | atg | agc | aag | ggg | agc | ctc | ctg | gat | ttc | ctg | aag | gga | gag | atg | 1173 |
| Glu | Tyr | Met | Ser | Lys | Gly | Ser | Leu | Leu | Asp | Phe | Leu | Lys | Gly | Glu | Met |      |
|     | 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |     |     |     |      |
| ggc | aag | tac | ctg | cgg | ctg | cca | cag | ctc | gtc | gat | atg | gct | gct | cag | att | 1221 |
| Gly | Lys | Tyr | Leu | Arg | Leu | Pro | Gln | Leu | Val | Asp | Met | Ala | Ala | Gln | Ile |      |
|     | 355 |     |     |     | 360 |     |     |     |     | 365 |     |     |     |     | 370 |      |
| gca | tcc | ggc | atg | gcc | tat | gtg | gag | agg | atg | aac | tac | gtg | cac | cga | gac | 1269 |
| Ala | Ser | Gly | Met | Ala | Tyr | Val | Glu | Arg | Met | Asn | Tyr | Val | His | Arg | Asp |      |
|     |     |     |     | 375 |     |     |     |     | 380 |     |     |     |     | 385 |     |      |
| ctg | cgg | gcg | gcc | aac | atc | ctg | gtg | ggg | gag | aac | ctg | gtg | tgc | aag | gtg | 1317 |
| Leu | Arg | Ala | Ala | Asn | Ile | Leu | Val | Gly | Glu | Asn | Leu | Val | Cys | Lys | Val |      |
|     |     |     | 390 |     |     |     |     | 395 |     |     |     |     | 400 |     |     |      |
| gct | gac | ttt | ggg | ctg | gca | cgc | ctc | atc | gag | gac | aac | gag | tac | aca | gca | 1365 |
| Ala | Asp | Phe | Gly | Leu | Ala | Arg | Leu | Ile | Glu | Asp | Asn | Glu | Tyr | Thr | Ala |      |
|     |     | 405 |     |     |     |     | 410 |     |     |     |     | 415 |     |     |     |      |
| cgg | caa | ggt | gcc | aag | ttc | ccc | atc | aag | tgg | aca | gcc | ccc | gag | gca | gcc | 1413 |
| Arg | Gln | Gly | Ala | Lys | Phe | Pro | Ile | Lys | Trp | Thr | Ala | Pro | Glu | Ala | Ala |      |
|     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |     |     |      |
| ctc | tat | ggc | cgg | ttc | acc | atc | aag | tcg | gat | gtc | tgg | tcc | ttc | ggc | atc | 1461 |
| Leu | Tyr | Gly | Arg | Phe | Thr | Ile | Lys | Ser | Asp | Val | Trp | Ser | Phe | Gly | Ile |      |
|     | 435 |     |     |     | 440 |     |     |     |     | 445 |     |     |     |     | 450 |      |
| ctg | ctg | act | gag | ctg | acc | acc | aag | ggc | cgg | gtg | cca | tac | cca | ggg | atg | 1509 |
| Leu | Leu | Thr | Glu | Leu | Thr | Thr | Lys | Gly | Arg | Val | Pro | Tyr | Pro | Gly | Met |      |
|     |     |     |     | 455 |     |     |     |     | 460 |     |     |     |     | 465 |     |      |
| gtc | aac | agg | gag | gtg | ctg | gac | cag | gtg | gag | agg | ggc | tac | cgc | atg | ccc | 1557 |
| Val | Asn | Arg | Glu | Val | Leu | Asp | Gln | Val | Glu | Arg | Gly | Tyr | Arg | Met | Pro |      |
|     |     |     | 470 |     |     |     | 475 |     |     |     |     |     | 480 |     |     |      |
| tgc | ccg | ccc | gag | tgc | ccc | gag | tcg | ctg | cat | gac | ctc | atg | tgc | cag | tgc | 1605 |
| Cys | Pro | Pro | Glu | Cys | Pro | Glu | Ser | Leu | His | Asp | Leu | Met | Cys | Gln | Cys |      |
|     |     | 485 |     |     |     |     | 490 |     |     |     |     | 495 |     |     |     |      |
| tgg | cgg | agg | gac | cct | gag | gag | cgg | ccc | act | ttt | gag | tac | ctg | cag | gcc | 1653 |

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Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu Gln Ala  
 500 505 510

ttc ctg gag gac tac ttc acc tcg aca gag ccc cag tac cag cct gga 1701  
 Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro Gln Tyr Gln Pro Gly  
 515 520 525 530

gag aac cta taggcctgga gctcctcctg gaccagagga ctcgctgtgg ggtacaggg 1759  
 Glu Asn Leu

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 <212> PRT  
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Gln Thr Pro Asn Lys Thr Ala Ala Pro Asp Thr His Arg Thr Pro Ser  
 35 40 45

Arg Ser Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe  
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Asn Thr Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala  
 65 70 75 80

Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr  
 85 90 95

Glu Thr Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn  
 100 105 110

Asn Thr Glu Gly Asp Trp Trp Leu Ala His Ser Leu Thr Thr Gly Gln  
 115 120 125

Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln  
 130 135 140

Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg  
 145 150 155 160

Leu Leu Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu  
 165 170 175

Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp  
 180 185 190

Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp  
 195 200 205

Ser Gly Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln  
 210 215 220

Gln Leu Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg  
 225 230 235 240

Leu Thr Asn Val Cys Pro Thr Ser Lys Pro Gln Thr Gln Gly Leu Ala  
 245 250 255

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Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys  
                   260                  265                  270  
 Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly  
                   275                  280                  285  
 Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro  
                   290                  295                  300  
 Glu Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Glu  
                   305                  310                  315                  320  
 Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile  
                   325                  330                  335  
 Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly  
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 Glu Met Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala  
                   355                  360                  365  
 Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His  
                   370                  375                  380  
 Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys  
                   385                  390                  395                  400  
 Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr  
                   405                  410                  415  
 Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu  
                   420                  425                  430  
 Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe  
                   435                  440                  445  
 Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro  
                   450                  455                  460  
 Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg  
                   465                  470                  475                  480  
 Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp Leu Met Cys  
                   485                  490                  495  
 Gln Cys Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu  
                   500                  505                  510  
 Gln Ala Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro Gln Tyr Gln  
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 Pro Gly Glu Asn Leu  
                   530

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&lt;223&gt; human c-SRC cDNA

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (134)..(1483)

&lt;400&gt; 4

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agctcctgag aag atg tca gca ata cag gcc gcc tgg cca tcc ggt aca 169
      Met Ser Ala Ile Gln Ala Ala Trp Pro Ser Gly Thr
      1             5             10

gaa tgt att gcc aag tac aac ttc cac ggc act gcc gag cag gac ctg 217
Glu Cys Ile Ala Lys Tyr Asn Phe His Gly Thr Ala Glu Gln Asp Leu
      15             20             25

ccc ttc tgc aaa gga gac gtg ctc acc att gtg gcc gtc acc aag gac 265
Pro Phe Cys Lys Gly Asp Val Leu Thr Ile Val Ala Val Thr Lys Asp
      30             35             40

ccc aac tgg tac aaa gcc aaa aac aag gtg ggc cgt gag ggc atc atc 313
Pro Asn Trp Tyr Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile
      45             50             55             60

cca gcc aac tac gtc cag aag cgg gag ggc gtg aag gcg ggt acc aaa 361
Pro Ala Asn Tyr Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys
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ctc agc ctc atg cct tgg ttc cac ggc aag atc aca cgg gag cag gct 409
Leu Ser Leu Met Pro Trp Phe His Gly Lys Ile Thr Arg Glu Gln Ala
      80             85             90

gag cgg ctt ctg tac ccg ccg gag aca ggc ctg ttc ctg gtg cgg gag 457
Glu Arg Leu Leu Tyr Pro Pro Glu Thr Gly Leu Phe Leu Val Arg Glu
      95             100             105

agc acc aac tac ccc gga gac tac acg ctg tgc gtg agc tgc gac ggc 505
Ser Thr Asn Tyr Pro Gly Asp Tyr Thr Leu Cys Val Ser Cys Asp Gly
      110             115             120

aag gtg gag cac tac cgc atc atg tac cat gcc agc aag ctc agc atc 553
Lys Val Glu His Tyr Arg Ile Met Tyr His Ala Ser Lys Leu Ser Ile
      125             130             135             140

gac gag gag gtg tac ttt gag aac ctc atg cag ctg gtg gag cac tac 601
Asp Glu Glu Val Tyr Phe Glu Asn Leu Met Gln Leu Val Glu His Tyr
      145             150             155

acc tca gac gca gat gga ctc tgt acg cgc ctc att aaa cca aag gtc 649
Thr Ser Asp Ala Asp Gly Leu Cys Thr Arg Leu Ile Lys Pro Lys Val
      160             165             170

atg gag ggc aca gtg gcg gcc cag gat gag ttc tac cgc agc ggc tgg 697
Met Glu Gly Thr Val Ala Ala Gln Asp Glu Phe Tyr Arg Ser Gly Trp
      175             180             185

gcc ctg aac atg aag gag ctg aag ctg ctg cag acc atc ggg aag ggg 745
Ala Leu Asn Met Lys Glu Leu Lys Leu Leu Gln Thr Ile Gly Lys Gly
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gag ttc gga gac gtg atg ctg ggc gat tac cga ggg aac aaa gtc gcc 793

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|             |       |       |      |        |            |       |       |       |       |            |      |     |     |      |            |      |
|-------------|-------|-------|------|--------|------------|-------|-------|-------|-------|------------|------|-----|-----|------|------------|------|
| Glu<br>205  | Phe   | Gly   | Asp  | Val    | Met<br>210 | Leu   | Gly   | Asp   | Tyr   | Arg<br>215 | Gly  | Asn | Lys | Val  | Ala<br>220 |      |
| gtc         | aag   | tgc   | att  | aag    | aac        | gac   | gcc   | act   | gcc   | cag        | gcc  | ttc | ctg | gct  | gaa        | 841  |
| Val         | Lys   | Cys   | Ile  | Lys    | Asn        | Asp   | Ala   | Thr   | Ala   | Gln        | Ala  | Phe | Leu | Ala  | Glu        |      |
|             |       |       |      | 225    |            |       |       |       | 230   |            |      |     |     | 235  |            |      |
| gcc         | tca   | gtc   | atg  | acg    | caa        | ctg   | cgg   | cat   | agc   | aac        | ctg  | gtg | cag | ctc  | ctg        | 889  |
| Ala         | Ser   | Val   | Met  | Thr    | Gln        | Leu   | Arg   | His   | Ser   | Asn        | Leu  | Val | Gln | Leu  | Leu        |      |
|             |       |       | 240  |        |            |       |       | 245   |       |            |      |     | 250 |      |            |      |
| ggc         | gtg   | atc   | gt   | gag    | gag        | aag   | ggc   | ggg   | ctc   | tac        | atc  | gtc | act | gag  | tac        | 937  |
| Gly         | Val   | Ile   | Val  | Glu    | Glu        | Lys   | Gly   | Gly   | Leu   | Tyr        | Ile  | Val | Thr | Glu  | Tyr        |      |
|             |       | 255   |      |        |            |       | 260   |       |       |            |      | 265 |     |      |            |      |
| atg         | gcc   | aag   | ggg  | agc    | ctt        | gtg   | gac   | tac   | ctg   | cgg        | tct  | agg | ggt | cgg  | tca        | 985  |
| Met         | Ala   | Lys   | Gly  | Ser    | Leu        | Val   | Asp   | Tyr   | Leu   | Arg        | Ser  | Arg | Gly | Arg  | Ser        |      |
|             | 270   |       |      |        |            | 275   |       |       |       |            | 280  |     |     |      |            |      |
| gtg         | ctg   | ggc   | gga  | gac    | tgt        | ctc   | ctc   | aag   | ttc   | tgc        | cta  | gat | gtc | tgc  | gag        | 1033 |
| Val         | Leu   | Gly   | Gly  | Asp    | Cys        | Leu   | Leu   | Lys   | Phe   | Ser        | Leu  | Asp | Val | Cys  | Glu        |      |
| 285         |       |       |      |        | 290        |       |       |       |       |            | 295  |     |     |      | 300        |      |
| gcc         | atg   | gaa   | tac  | ctg    | gag        | ggc   | aac   | aat   | ttc   | gtg        | cat  | cga | gac | ctg  | gct        | 1081 |
| Ala         | Met   | Glu   | Tyr  | Leu    | Glu        | Gly   | Asn   | Asn   | Phe   | Val        | His  | Arg | Asp | Leu  | Ala        |      |
|             |       |       |      | 305    |            |       |       |       | 310   |            |      |     |     | 315  |            |      |
| gcc         | cgc   | aat   | gtg  | ctg    | gtg        | tct   | gag   | gac   | aac   | gtg        | gcc  | aag | gtc | agc  | gac        | 1129 |
| Ala         | Arg   | Asn   | Val  | Leu    | Val        | Ser   | Glu   | Asp   | Asn   | Val        | Ala  | Lys | Val | Ser  | Asp        |      |
|             |       |       | 320  |        |            |       |       | 325   |       |            |      |     | 330 |      |            |      |
| ttt         | ggt   | ctc   | acc  | aag    | gag        | gcg   | tcc   | agc   | acc   | cag        | gac  | acg | ggc | aag  | ctg        | 1177 |
| Phe         | Gly   | Leu   | Thr  | Lys    | Glu        | Ala   | Ser   | Ser   | Thr   | Gln        | Asp  | Thr | Gly | Lys  | Leu        |      |
|             |       | 335   |      |        |            |       | 340   |       |       |            |      | 345 |     |      |            |      |
| cca         | gtc   | aag   | tgg  | aca    | gcc        | cct   | gag   | gcc   | ctg   | aga        | gag  | aag | aaa | ttc  | tcc        | 1225 |
| Pro         | Val   | Lys   | Trp  | Thr    | Ala        | Pro   | Glu   | Ala   | Leu   | Arg        | Glu  | Lys | Lys | Phe  | Ser        |      |
|             | 350   |       |      |        |            | 355   |       |       |       |            | 360  |     |     |      |            |      |
| act         | aag   | tct   | gac  | gtg    | tgg        | agt   | ttc   | gga   | atc   | ctt        | ctc  | tgg | gaa | atc  | tac        | 1273 |
| Thr         | Lys   | Ser   | Asp  | Val    | Trp        | Ser   | Phe   | Gly   | Ile   | Leu        | Leu  | Trp | Glu | Ile  | Tyr        |      |
| 365         |       |       |      |        | 370        |       |       |       | 375   |            |      |     |     | 380  |            |      |
| tcc         | ttt   | ggg   | cga  | gtg    | cct        | tat   | cca   | aga   | att   | ccc        | ctg  | aag | gac | gtc  | gtc        | 1321 |
| Ser         | Phe   | Gly   | Arg  | Val    | Pro        | Tyr   | Pro   | Arg   | Ile   | Pro        | Leu  | Lys | Asp | Val  | Val        |      |
|             |       |       |      | 385    |            |       |       |       | 390   |            |      |     |     | 395  |            |      |
| cct         | cgg   | gtg   | gag  | aag    | ggc        | tac   | aag   | atg   | gat   | gcc        | ccc  | gac | ggc | tgc  | cgg        | 1369 |
| Pro         | Arg   | Val   | Glu  | Lys    | Gly        | Tyr   | Lys   | Met   | Asp   | Ala        | Pro  | Asp | Gly | Cys  | Pro        |      |
|             |       |       | 400  |        |            |       |       | 405   |       |            |      |     | 410 |      |            |      |
| ccc         | gca   | gtc   | tat  | gaa    | gtc        | atg   | aag   | aac   | tgc   | tgg        | cac  | ctg | gac | gcc  | gcc        | 1417 |
| Pro         | Ala   | Val   | Tyr  | Glu    | Val        | Met   | Lys   | Asn   | Cys   | Trp        | His  | Leu | Asp | Ala  | Ala        |      |
|             |       | 415   |      |        |            | 420   |       |       |       |            |      | 425 |     |      |            |      |
| atg         | cgg   | ccc   | tcc  | ttc    | cta        | cag   | ctc   | cga   | gag   | cag        | ctt  | gag | cac | atc  | aaa        | 1465 |
| Met         | Arg   | Pro   | Ser  | Phe    | Leu        | Gln   | Leu   | Arg   | Glu   | Gln        | Leu  | Glu | His | Ile  | Lys        |      |
|             | 430   |       |      |        | 435        |       |       |       | 440   |            |      |     |     |      |            |      |
| acc         | cac   | gag   | ctg  | cac    | ctg        | tga   | cgg   | cgtg  | cctc  | cgc        | cctg | ggt | cat | gggc |            | 1513 |
| Thr         | His   | Glu   | Leu  | His    | Leu        |       |       |       |       |            |      |     |     |      |            |      |
| 445         |       |       |      |        | 450        |       |       |       |       |            |      |     |     |      |            |      |
| ctgtgggggac | tgaac | ctgga | agat | catgga | cctgg      | tgccc | ctgct | cactg | ggccc | gagacc     |      |     |     |      |            | 1573 |

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 aagtacgaat cttatttttc ctgtcctgcc cgtgaggggtg gggggggaccg ggccccctctc 1933  
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 Gly Asp Val Leu Thr Ile Val Ala Val Thr Lys Asp Pro Asn Trp Tyr  
 35 40 45  
 Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile Pro Ala Asn Tyr  
 50 55 60  
 Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys Leu Ser Leu Met  
 65 70 75 80  
 Pro Trp Phe His Gly Lys Ile Thr Arg Glu Gln Ala Glu Arg Leu Leu  
 85 90 95  
 Tyr Pro Pro Glu Thr Gly Leu Phe Leu Val Arg Glu Ser Thr Asn Tyr  
 100 105 110  
 Pro Gly Asp Tyr Thr Leu Cys Val Ser Cys Asp Gly Lys Val Glu His  
 115 120 125  
 Tyr Arg Ile Met Tyr His Ala Ser Lys Leu Ser Ile Asp Glu Glu Val  
 130 135 140  
 Tyr Phe Glu Asn Leu Met Gln Leu Val Glu His Tyr Thr Ser Asp Ala  
 145 150 155 160  
 Asp Gly Leu Cys Thr Arg Leu Ile Lys Pro Lys Val Met Glu Gly Thr  
 165 170 175  
 Val Ala Ala Gln Asp Glu Phe Tyr Arg Ser Gly Trp Ala Leu Asn Met

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| 180 |     |     |     |     |     |     |     |     |     | 185 |     |     |     |     | 190 |  |  |  |  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|--|--|
| Lys | Glu | Leu | Lys | Leu | Leu | Gln | Thr | Ile | Gly | Lys | Gly | Glu | Phe | Gly | Asp |  |  |  |  |
|     |     | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |  |  |  |  |
| Val | Met | Leu | Gly | Asp | Tyr | Arg | Gly | Asn | Lys | Val | Ala | Val | Lys | Cys | Ile |  |  |  |  |
|     | 210 |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |  |  |  |  |
| Lys | Asn | Asp | Ala | Thr | Ala | Gln | Ala | Phe | Leu | Ala | Glu | Ala | Ser | Val | Met |  |  |  |  |
|     | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     | 240 |  |  |  |  |
| Thr | Gln | Leu | Arg | His | Ser | Asn | Leu | Val | Gln | Leu | Leu | Gly | Val | Ile | Val |  |  |  |  |
|     |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |  |  |  |  |
| Glu | Glu | Lys | Gly | Gly | Leu | Tyr | Ile | Val | Thr | Glu | Tyr | Met | Ala | Lys | Gly |  |  |  |  |
|     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |     |  |  |  |  |
| Ser | Leu | Val | Asp | Tyr | Leu | Arg | Ser | Arg | Gly | Arg | Ser | Val | Leu | Gly | Gly |  |  |  |  |
|     |     | 275 |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |  |  |  |  |
| Asp | Cys | Leu | Leu | Lys | Phe | Ser | Leu | Asp | Val | Cys | Glu | Ala | Met | Glu | Tyr |  |  |  |  |
|     | 290 |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     |  |  |  |  |
| Leu | Glu | Gly | Asn | Asn | Phe | Val | His | Arg | Asp | Leu | Ala | Ala | Arg | Asn | Val |  |  |  |  |
|     | 305 |     |     |     | 310 |     |     |     |     |     | 315 |     |     |     | 320 |  |  |  |  |
| Leu | Val | Ser | Glu | Asp | Asn | Val | Ala | Lys | Val | Ser | Asp | Phe | Gly | Leu | Thr |  |  |  |  |
|     |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |  |  |  |  |
| Lys | Glu | Ala | Ser | Ser | Thr | Gln | Asp | Thr | Gly | Lys | Leu | Pro | Val | Lys | Trp |  |  |  |  |
|     |     |     | 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |     |  |  |  |  |
| Thr | Ala | Pro | Glu | Ala | Leu | Arg | Glu | Lys | Lys | Phe | Ser | Thr | Lys | Ser | Asp |  |  |  |  |
|     |     | 355 |     |     |     |     | 360 |     |     |     |     | 365 |     |     |     |  |  |  |  |
| Val | Trp | Ser | Phe | Gly | Ile | Leu | Leu | Trp | Glu | Ile | Tyr | Ser | Phe | Gly | Arg |  |  |  |  |
|     | 370 |     |     |     |     | 375 |     |     |     |     | 380 |     |     |     |     |  |  |  |  |
| Val | Pro | Tyr | Pro | Arg | Ile | Pro | Leu | Lys | Asp | Val | Val | Pro | Arg | Val | Glu |  |  |  |  |
|     | 385 |     |     |     | 390 |     |     |     |     |     | 395 |     |     |     | 400 |  |  |  |  |
| Lys | Gly | Tyr | Lys | Met | Asp | Ala | Pro | Asp | Gly | Cys | Pro | Pro | Ala | Val | Tyr |  |  |  |  |
|     |     |     |     | 405 |     |     |     |     | 410 |     |     |     |     | 415 |     |  |  |  |  |
| Glu | Val | Met | Lys | Asn | Cys | Trp | His | Leu | Asp | Ala | Ala | Met | Arg | Pro | Ser |  |  |  |  |
|     |     |     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |  |  |  |  |
| Phe | Leu | Gln | Leu | Arg | Glu | Gln | Leu | Glu | His | Ile | Lys | Thr | His | Glu | Leu |  |  |  |  |
|     |     | 435 |     |     |     |     | 440 |     |     |     |     | 445 |     |     |     |  |  |  |  |
| His | Leu |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |  |  |  |
|     | 450 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |  |  |  |

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&lt;211&gt; 14

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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epitope tag

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